Conserved gut microbiota in a herbivorous beetle mediates the degradation of host plant defenses

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To my parents, my absolute role model.

To my sister, my best friend.

To Hassan, for his unconditional support.

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LIST OF PUBLICATIONS

This thesis is based in the following manuscripts:

Nagel, R., Berasategui, A., Paetz, C., Gershenzon, J., Schmidt, A. (2014)
Overexpression of an isoprenyl diphosphate synthase in spruce leads to unexpected terpene diversion products that function in plant defense.
Plant Physiology (Chapter 2)

Berasategui, A., Axelsson, A., Norlander, G., Borg-Karlson, A-K., Schmidt, A., Gershenzon, J., Terenius, O., Kaltenpoth, M. (2016)
The gut microbiota of the pine weevil is similar across Europe and resembles that of other conifer-feeding beetles.
Molecular Ecology (Chapter 3)

Dohet L., Gregoire J.C., Berasategui A., Kaltenpoth M., Biedermann P.H.W. (2016)
Bacterial and fungal symbionts of parasitic *Dendroctonus* bark beetles.
FEMS Microbiolgy and Ecology (Chapter 4)

Berasategui A., Salem, H., Paetz, C., Schmidt, A., Kaltenpoth, M., Gershenzon J. (In preparation)
Conserved microbiota in conifer-feeding beetles mediates the degradation of host plant defenses in the pine weevil.
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Potential applications of insect symbionts in biotechnology.
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CHAPTER I:

INSECT HERBIVORY IN TOXIC PLANTS

1.1. PLANT-INSECT INTERACTIONS: AN ARMS-RACE

Insects and plants represent 73% of all known species on Earth (Roskov *et al.* 2016) and have been interacting with each other for millions of years (Labandeira and Currano 2013). Although some of those interactions, such as pollination, are beneficial for both partners, others, such as herbivory, are not. Ehrlich and Raven (1964) in their co-evolutionary hypothesis proposed that antagonistic insect-plant interactions could drive a major part of the biodiversity observed in nature. They reasoned that any plant species may evolve a defensive strategy in response to herbivory that deters or kills insect herbivores, thereby escaping most damage. The absence of herbivory could allow a plant population to persist and broaden the range of habitats it can exploit, favoring opportunities for local adaptation, genetic drift and speciation (Futuyma 2000, Weber *et al.* 2014), which could lead to the radiation and diversification of the plant lineage. Eventually, however, herbivores will adapt to such defended plants by evolving a counter adaptation against the plant's defenses. Like most adaptations, including plant's defenses, these have often been regarded as a mutation or recombination event (Despres *et al.* 2007, Edger *et al.* 2015, but see section 1.4). This innovation allows the insect to exploit new ecological niches, which may again lead to the radiation and diversification of the insect herbivore population. This co-evolving process has been referred to as an arms race. Thus, insects drive the evolution of plant chemical defenses, and these, in turn, drive the evolution of insect counter adaptations (Ehrlich and Raven 1964).

1.2. PLANT SECONDARY METABOLITES AS PLANT DEFENSES

Plants are constantly challenged by herbivores both above and belowground. With their limited ability to escape, plants have evolved numerous defense strategies to overcome these attacks (Lucas *et al.* 2000, Mithoefer and Boland 2012). Many plants have evolved thorns, spines or lignified structures to avoid or minimize herbivory (Fernandes 1994). However, in addition to these structural defenses, they have also evolved chemical defenses (Fürstenberg-Hägg *et al.* 2013). Karban and Baldwin (1997) defined these molecules as "compounds that increase plant fitness in the presence, but not absence of herbivores". These substances include many of what are known as plant secondary metabolites, since they are, for the most part, not involved in primary metabolism (Pichersky and Lewinsohn 2011). Although not all secondary metabolites participate in plant protection (Gershenzon and Dudareva 2007, Pichersky and Lewinsohn 2011), for many of these compounds, defense against pathogens, competitors or predators is their only plausible role (Agrawal and Konno 2009). Hence, throughout this body of work, we will use the terms "plant secondary metabolite" and "plant chemical defense" interchangeably.

Chemical defenses can be classified as constitutive or induced (Kaplan *et al.* 2008, Keeling and Bohlmann 2006). Constitutive defenses are always present in a plant, normally in tissues that are attacked often (Wittstock and Gershenzon 2002), whereas induced ones are produced only upon attack (Stotz *et al.* 2000). Likewise, plant secondary metabolites can be classified as indirect and direct defenses (Kant *et al.* 2004). Direct chemical defenses often act as deterrents and can be toxic to insects through a variety of deleterious effects, hindering the normal functioning of insect metabolism (Mithoefer and Boland 2012). Indirect defenses, on the other hand, are used to attract herbivore natural enemies such as predators and parasitoids (Delphia *et al.* 2007).

The diversity in chemical structure of plant secondary metabolites is extraordinary and includes compounds classified as alkaloids, benzoxazinoids, cyanogenic glycosides, glucosinolates, phenolics, and terpenes (Fürstenberg-Hägg *et al.* 2013). Their mode of action is, in most cases, unknown but some disrupt gut membranes, impede normal nutrient and ion transport, hinder metabolism and signaling processes or interrupt of hormone-controlled physiological processes (Mithoefer and Boland 2012). Some compounds have a very specific target, such as cardenolides, present in milkweeds, which exert their toxicity through inhibition of normal neurotransmission by targeting the Na-K ATPases of animals that regulate electric potential in cells (Agrawal *et al.* 2012). Other compounds, such as glucosinolates, have a more general mode of action (Jeschke *et al.* 2015). As an activated defense, glucosinolates are compartmentalized, separated from their hydrolyzing enzyme, the myrosinase. Upon herbivory, cells are broken and glucosinolates and myrosinases come into contact, which results in the cleavage of the glycoside molecule. The resulting aglycone then rearranges into nitriles or isothiocyanates. The latter spontaneously react with a variety of nucleophiles present in cells, inactivating proteins, and nucleic acids (Jeschke *et al.* 2015). Given their toxicity and wide distribution, plant secondary metabolites exert a strong selective pressure on insect herbivores to overcome them.

1.3. INSECT COUNTER-ADAPTATIONS TO PLANT DEFENSES

As presented above, most previous research on plant defensive chemistry has been dedicated towards elucidating the structural diversity of secondary metabolites in plants rather than their mode of action. Consequently, little is known about the counter-adaptations insects have evolved (Despres *et al.* 2007). Broadly speaking, insect counter-adaptations are involved in the avoidance of plant toxins, their excretion, sequestration, the acquisition of metabolic resistance, and the evolution of target site mutations (Despres *et al.* 2007).

Herbivorous insects may try to avoid eating toxic plants by consuming a plant at a stage in which chemical defenses are low or by foraging on organs that do not contain the toxin (Nealis *et al.* 2005). For example, tiger moths feed exclusively on the upper section of conifer needles that are lower in chemical defenses than the basal fraction (Litvak and Monson 1998). Many plants store chemical defenses under pressure in channels such as resin ducts in conifers or laticifers in latex-producing plants (Farrel *et al.* 1991). Accordingly, several insect orders have evolved behaviors to disrupt those channels and avoid the release of plant defenses at the feeding site (Dussourd 1994, Dussourd and Denno 1993). Among the Orthoptera, Coleoptera and Lepidoptera, various species have independently evolved vein cutting and trenching behaviors in latex-containing plants (Agrawal 2009). Likewise, Blepharida beetles sever their host plant leaves before consuming them (Becerra 1994), and sawflies (Diprionidae) damage resin ducts of conifers to release chemical defenses before feeding (McCullough and Wagner 1993). Lastly, insects could also try to prevent the synthesis of plant toxins (Bede *et al.* 2006). Oral secretions of chewing

insects such as caterpillars, contain salivary elicitors, molecules that inhibit or reduce plant secondary metabolite synthesis (Musser *et al.* 2002).

Metabolic resistance

Some insects can metabolize plant secondary metabolites through the production of detoxification enzymes such as P450s, glutathion-S-transferases, UDP-glycosyltransferases and carboxylases (Despres *et al.* 2007). P450s catalyze more than 60 different reactions with a wide capacity to degrade an array of plant secondary compounds (Feyereisen 2012). Glutathione-S-transferases are often involved in insect resistance against glucosinolates (Francis *et al.* 2005), and catalyze the conjugation of glutathione to electrophilic molecules, which renders toxins more soluble and easily excreted (Enayati *et al.* 2005). For carboxylesterases, on the other hand, there is little evidence regarding their role in insect counter-adaptations to plant defenses (Despres *et al.* 2007). However, their involvement in processing insecticides suggests that they could be involved in plant toxin degradation as well (Yang *et al.* 2005). Lastly, UDP-glycosyltransferases catalyze the conjugation of a sugar to other molecules, increasing their polarity and facilitation their excretion (Ahn *et al.* 2012).

Excretion, sequestration, and further use of plant toxins

Although insects normally excrete plant secondary metabolites after modifying them (Francis *et al.* 2005, Ahn *et al.* 2012), sometimes they can rapidly excrete them unmodified before any harm is done (Kennedy and Tierney 2013). Likewise, some insects can excrete a great fraction of ingested chemical defenses during molting (Zragobelny *et al.* 2004). Additionally, some herbivores can sequester plant toxins and use them for their own protection against UV light (Carrol *et al.* 1997), or more commonly against predators by making their tissues unpalatable (Ode 2006). Little is known about the mechanistic basis of sequestration in insects (Petschenka and Agrawal 2016). In general, the plant toxin present in the gut lumen has to cross the gut wall to the hemolymph and then must be transferred to the reservoir tissue (Kuhn *et al.* 2004, Erb and Robert 2016). In some leaf beetles (Chrysomelidae) the concentration of toxic glucosides is higher in the gut than in the hemolymph, and so the transport occurs by diffusion and is likely quite unspecific (Kuhn *et al.* 2004). A second transport system transfers the glucoside from the hemolymph to the reservoir through a specific ABC transporter (Strauss *et al.* 2013, Discher *et al.* 2009). Given that a number of herbivores accumulate various glucosides in the hemolymph, a highly specific transport mechanism is likely involved in movement to the reservoir (Erb and Robert 2016). Likewise, identical compounds can be sequestered by different physiological mechanisms in different insects (Petschenka and Agrawal 2016).

Target site mutation

Insects can also evolve resistance against plant secondary metabolites through mutations in the target site of plant toxins (Berenbaum 1986). Cardenolides exert their toxicity by binding to the Na+/K+ -ATPase of animals, hindering normal ion transport, causing membrane instability and impeding active transport among other effects (Agrawal *et al.* 2012). Several different milkweed-feeding insect lineages have acquired amino acid substitutions in the target site of cardenolides (Dobler *et al.* 2012). These substitutions modify the configuration of the binding pocket of the Na+/K+ -ATPase, thereby reducing

the affinity of cardenolides for the ATP pump and conferring insects with resistance (Petschenka *et al.* 2013).

While the complexity of some plant defense systems as well as their diverse modes of action has resulted in the evolution of a wide range of counter adaptations in insect herbivores (i.e. glucosinolates, see Jeschke *et al.* 2015), other metabolites (cardenolides) with a very specific mode of action, have exerted a strong selective pressure on insects to evolve a narrow range of adaptations (Whiteman and Mooney 2012). However, individual herbivore counter-adaptations against plant defenses are often not completely effective (Parr and Thurston 1972; Agrawal *et al.* 2012; Richards *et al.* 2012) and insects must combine more than one. This is the case for monarch butterfly caterpillars which combine four different strategies to cope with milkweed chemical defenses, namely vein cutting, sequestration, metabolism of toxins as well as target site mutation (Despres *et al.* 2007).

1.4. Role of symbiotic microbes as counter-adaptations against plant defense

Traditionally, insect counter-adaptations aimed at mitigating the deleterious effects of plant toxins were thought to be primarily encoded in an insect's genome (Despres *et al.* 2007, Zhu-Salzman *et al.* 2005). However, there is overwhelming evidence that mutualistic microbes can mediate host plant use by supplementing nutrients to their insect hosts, degrading complex plant polymers such as lignin or cellulose (Douglas 2009). Recent data suggest that symbiotic microbes could also interfere with plant defenses (Hammer and Bowers 2015).

Microbial symbionts are able to benefit their host by manipulating plant defenses or preventing the synthesis of compounds targeting insects (Chung *et al.* 2013, Barr *et al.* 2010). Many insects contain in their oral secretions molecules known as herbivore-associated molecular patterns (HAMPs) that are recognized by the host plant and trigger anti-herbivore responses (Mithöfer and Boland 2008). As a counter adaptation, some insects have evolved proteins that reduce or inhibit plant defense (Consales *et al.* 2012). In an analogous manner, Colorado potato beetle (*Leptinotarsa decemlineata*) larvae feeding on tomato plants use symbiotic bacteria in their oral secretion to suppress the synthesis of chemical defensive compounds (Chung *et al.* 2013). The presence of bacteria misleads the plant in perceiving the attack as microbial instead of herbivorous. The absence of toxic compounds benefits beetle larvae whose growth is increased compared to those treated with antibiotics (Chung *et al.* 2013). Another example suggests that western corn beetles (*Diabrotica virgifera virgifera*) infected with *Wolbachia* are able to down-regulate genes involved in defense in their maize host plants through an unknown mechanism; whereas uninfected beetles cannot (Barr *et al.* 2010). Robert and colleagues (2013) on the other hand, found no effect of *Wolbachia* on the performance of *D. virgifera* feeding on maize.

Once plant defenses have been synthesized, however, insects must employ different mechanisms to overcome them (Despres *et al.* 2007). In this instance, a strategy would be to physically avoid the toxicity of these compounds. Some plant defenses require hydrolyzing enzymes in order to become active (Pentzold *et al.* ,2014). Microbial symbionts could synthesize compounds that compete for the binding site of these enzymes (Hammer and Bowers 2015), preventing cleavage of the target molecule and avoiding toxicity. Although this has not been reported in insects, a similar process has been described in human gut bacteria (Clayton *et al.* 2009). Interestingly, the production of amino acids by symbionts, which has traditionally been viewed as playing a nutritional role, could also play a protective function

against plant defenses (Hammer and Bowers 2015). Konno and colleagues (1997, 1998) described that the amino acid glycine in the gut of a lepidopteran larva could attach to toxic polyphenols, inactivating them and reducing or avoiding toxicity.

Another strategy to lower noxious effects of plant secondary metabolites is symbiont-mediated break-down of these compounds (Hammer and Bowers 2015, Despres *et al.* 2007). Larvae of the cabbage root fly (*Delia radicum*) feed on Brassica plants, highly toxic due to the high amount of glucosinolates (GLS) (Hopkins *et al.* 2009). Upon disruption of plant tissues, GLS and the hydrolyzing enzyme myrosinase come into contact, leading to the cleavage and activation of GLS and the production of high amounts of

toxic isothiocyanates, among other compounds (reviewed in Jeschke *et al.* 2015). Several members of the microbial community harbored by the cabbage root fly contain plasmids carrying the *saxA* gene that allows them to degrade isothiocyanates (Welte *et al.* 2015). Likewise, the coffee bean borer (*Hypothenemus hampei*) harbors a core microbiota across geographical regions and coffee plant species, that is able to degrade the alkaloid caffeine (Ceja-Navarro *et al.* 2015). This compound is toxic to many insects and is thought to be involved in plant defense (Nathanson *et al.* 1984). Treatment with antibiotics disrupts caffeine degrading activity, with subsequent effects on host fitness, such as lower fecundity and hatching rates (Ceja-Navarro *et al.* 2015). Reinfection with a *Pseudomonas* strain, rescues the degrading phenotype, demonstrating that the microbial community is responsible for caffeine degradation and that *Pseudomonas* alone plays a crucial role (Ceja-Navarro *et al.* 2015). In addition to glucosinolates and alkaloids, many other plant defenses are degraded by insect microbial symbionts: tannins (DeFine Licht *et al.* 2013), phenolics (Hammerbacher *et al.* 2013), flavonoids (Lauzon *et al.* 2003) and salicinoids (Mason *et al.* 2014).

The mode of action of many plant defenses lies in their ability to disrupt gut membranes and lipidic bilayers (De Geyter *et al.* 2012). Thus, if the aforementioned strategies are not utilized, mutualistic microbes could still benefit their host by preventing the absorption of plant toxins through the gut wall. *Enterococcus casseliflavus* harbored in the gut of lepidopteran larvae of *Spodoptera litura*, forms a biofilm that causes the crystallization of toxic terpenes (Shao *et al.* 2011), precluding them from crossing the gut epithelium. Failure to establish such a biofilm leads to higher mortality in moth larvae (Shao *et al.* 2011).

Microbial degradation of plant defenses is not unique to insects. Among vertebrates, herbivorous mammals such as cows, sheep and rats, as well as birds are known to harbor microbial communities able to detoxify plant toxins (Miller *et al.* 2014; Kohl and Dearing 2012, Garcia-Amado *et al.* 2007, Kohl *et al.* 2016). Thus, there is growing evidence demonstrating that associations with symbiotic organisms to manipulate and degrade plant defenses are substantially more common than previously thought (Shen and Dowd 1990, Kikuchi *et al.* 2012, Welte *et al.* 2015, Ceja-Navarro *et al.* 2015, Boone *et al.* 2013, Chung *et al.* 2013) and might well be widespread.

1.5. WHY DELEGATE TO MICROBES?

Some organisms are unable to perform certain functions or produce particular compounds due to phylogenetic or metabolic constraints. Differences in the genetic load of two organisms could be explained because their lineages diverged before a particular trait evolved (Moran 2007). For example, all phototrophs such as Chlorobi, purple bacteria or cyanobacteria can capture light to produce ATP;

whereas oxygenic photosynthesis evolved within the cyanobacterial lineage (Mulkidjanian *et al.* 2006). Hence, the machinery required for photosynthesis must have evolved once these lineages diverged. Alternatively, a particular function might have arisen before the divergence of two lineages and have secondarily been lost in one of them. This is the case of many metabolic pathways that have been lost in the animal kingdom (Moran 2007). For instance, the pathway leading to the synthesis of tryptophan has evolved only once, and did so before the different domains of life diverged (Yanovsky 2007). However, this pathway has been lost in all animals and many other organisms thus rendering them unable to synthetize this amino acid (Payne and Loomis 2006). Usually, pathways that are lost are those with many enzymatic steps that are energetically costly (Moran 2007). This suggests that if the product needed is readily available in the environment, natural selection will favor inactivation or elimination of the pathway that produces it (Moran 2007).

Organisms that lack particular metabolic pathways can thus acquire the necessary product from the environment, through horizontal gene transfer, or through symbiosis (Moran 2007). For example, no animal to date has re-evolved the ability to synthesize tryptophan and most acquire this essential amino acid from their diet (Yanovsky 2007). Others, such as cicadas associate with *Sulcia mulleri*, a bacterium that is able to supplement the insect diet with tryptophan as well as nine other amino acids (McCutcheon *et al.* 2009). Likewise, plants are dependent on nitrogen for growth but have never evolved the ability to fix atmospheric nitrogen. In turn, they have repeatedly evolved symbiotic interactions with nitrogen-fixing bacteria (Franche *et al.* 2009). Therefore, in some circumstances, evolving a symbiotic interaction may be easier than evolving or re-evolving a metabolic function.

Microorganisms possess unique traits that allow them to cope with plant toxins and complement the resistant traits of insect herbivores (Hammer and Bowers). First, microbes, have a metabolic diversity that allows them to degrade countless different chemicals and use many of them as carbon sources (Laskin and Lechevalier 1984). Some microbes are unique in their ability to completely mineralize plant chemicals such as alkaloids, phenolics and glucosinolates (Boll *et al.* 2014). Second, microbes have short generation periods which allow for rapid changes in microbial communities upon environmental fluctuations (Letourneau 1988). In keeping with this fact, changes in an herbivore diet can alter the gut microbiota in a short period of time (David *et al.* 2014). Plant secondary metabolites could promote the growth of bacteria able to degrade them (Hammer and Bowers 2015). For instance, the addition of certain phenolic compounds to the diet of woodrats dramatically changes their gut bacterial community structure towards one rich in genes involved in the degradation of these substances (Kohl *et al.* 2014). Third, the ease with which microbes undergo horizontal gene transfer can lead to the rapid spread of traits in microbial populations. For example, genes coding for enzymes degrading algal polysaccharides are known to have been transferred from alga-associated microbes to the gut microbiota of Japanese people (Hehemann *et al.* 2010, 2012).

Despite the many benefits engaging in a symbiotic interaction presents, mutualisms are not exempt from conflict (Bennet and Moran 2015). Acquiring and maintaining a symbiotic association can be costly (Vigneron *et al.* 2014). Providing shelter, nutrients and ensuring symbiont transmission can be energetically expensive (Bennet and Moran 2015, Salem *et al.* 2015). Also, the host must ensure the symbiont does not spread, invading the whole body (Login *et al.* 2011). However, given how ubiquitous symbioses are in nature (McFall Ngai *et al.* 2013) and how frequently similar associations evolve (Bittleston *et al.* 2016), the benefits these interactions provide must outweigh the costs in many circumstances.

1.6. CONIFERS AND CONIFER-FEEDING BEETLES AS A MODEL TO STUDY HOST-MICROBE INTERACTIONS

Despite the great diversity of plant-insect interactions, not every system is equally suitable to the study of symbiont-mediated degradation of plant secondary metabolites. Conifers and conifer-feeding beetles, however, represent an exciting and promising system for several reasons. First, conifer chemistry has been extensively studied. Specifically, the biosynthesis and catabolism of a number of defensive compounds are well known (Franceschi 2005, Phillips 1999). Second, beetles, as chewing herbivores, are more exposed to plant chemical defenses than, for instance, sap-feeding insects given that they damage and ingest large portions of plant tissues (Mullin 1986). Lastly, as opposed to Lepidopterans harboring simple transient microbiotas with no known beneficial function (Whitaker *et al.* 2016), beetles can form consistent interactions with microbes (Toju *et al.* 2013, Kölsch *et al.* 2009) many of which are beneficial (Kuriwada *et al.* 2010).

1.6.1. Conifers and conifer defenses

Conifers, the largest plant group within the gymnosperms, evolved during the Carboniferous period, around 300 million years ago (Henry 2005). Although the number of species is relatively low (around 600) (Farjon *et al.* 1999), they are extremely important from an ecological and economical point of view. Conifers are known for their production of resin, although not all families produce it and only the Pinaceae and Araucariaceae families do so in large amounts (Langenheim 2003). Resin as defined by Langenheim (2003) is a complex mixture of volatile and non-volatile terpenoids and phenolic secondary metabolites. It is secreted in specialized structures located inside or on the surface of the plant and has a potential role in ecological interactions (Keeling and Bohlmann 2006).

With more than 30,000 described compounds, terpenes are the largest class of plant secondary metabolites described to date, although they can also be involved in primary metabolism (Trapp and Croteau 2001). As primary metabolites, terpenoids are involved in maintaining organismal integrity and normal vital functions. For instance, sterols are essential for cell membrane integrity (Dufourc 2008) or can act as hormones in animals such as the juvenile hormone in insects (Bellés *et al.* 2005). Likewise, carotenoids, pigments involved in photosynthesis (Cazzonelli and Pogson 2010) are also of a terpenoid nature.

As secondary metabolites terpenes are often involved in defense (Gershenzon and Dudareva 2007), increasing plant fitness under biotic or abiotic stress. As defensive compounds against herbivores, terpenes can function in indirect as well as direct plant defenses (Huber and Bohlmann 2006), and although they are often constitutively expressed, they can also be induced upon herbivore attack (Litvak and Monson 1998).

Terpene biosynthesis

The metabolic pathway encoding terpene biosynthesis is highly conserved (Lombard and Moreirra 2011). Isopentenyl diphosphate (IDP) and dimethylallyl diphosphate (DMADP), terpene initial building blocks, are synthetized by two pathways, the mevalonate (MVA), and methylerythritol phosphate (MEP) pathways. The MVA pathway occurs in eukaryotes and some bacteria, while the MEP pathway is present

in both bacteria and photosynthetic eukaryotes (Lombard and Moreirra, 2011, Hemmerlin *et al.* 2012). Both pathways are present in plants, where the MVA pathway is located in the cytosol and the MEP pathway in the plastids (Hemmerlin *et al.* 2012). The MVA pathway produces only IDP, whereas the MEP pathway produces both IDP and DMADP (Hemmerlin *et al.* 2012).

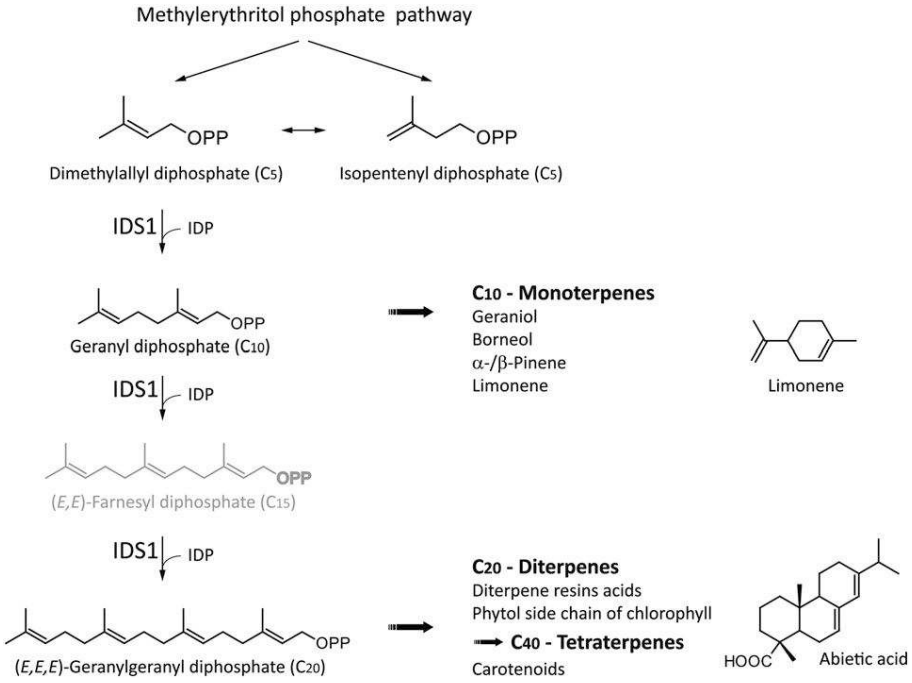

Figure 1. Outline of terpenoid biosynthesis. The diagram depicts the reactions carried out by the IDS1 from *P. abies*. The 5 carbon building blocks IDP and DMADP, synthesized in the MEP pathway, are the substrates for IDS1. Successive condensations of DMADP with one to three molecules of IDP mediated by IDS form GDP, FDP, or GGDP. The different prenyl diphosphates are then converted into members of the different terpene classes: GDP to monoterpenes and GGDP to diterpenes and tetraterpenes. FDP is produced by IDS1 as an intermediate but is not released. OPP, diphosphate moiety. (From Nagel *et al.* 2014)

Most terpenes are synthesized from IDP through a set of reactions catalyzed by isoprenyl diphosphate synthases (IDS) enzymes (Figure 1). These enzymes mediate the successive alkylation of different numbers of IPP (C5) units by an allylic diphosphate, normally DMADP (C5), producing geranyl diphosphate (GDP, C10), farnesyl diphosphate (FDP, C15) and geranylgeranyl diphosphate (GGDP, C20). These compounds are the precursors of mono-, sesqui- and diterpenes respectively (Ohnuma *et al.* 1998; Dewick, 2002). Six different IDSs have been identified in conifers (Martin *et al.* 2004; Schmidt and Gershenzon 2007 and 2008; Schmidt *et al.* 2011), with IDS1 producing both GDP and GGDP, the precursor of mono- and diterpenes respectively. Given that conifer resin is mainly composed of mono- (C10) and diterpenes (C20), with minor amounts of sesquiterpenes (Martin *et al.* 2002), IDS1 represents an integral enzyme for terpene biosynthesis. Changes in the expression of different enzymes of terpene biosynthesis (such as IDSs), can lead to changes in terpene content (Banyai *et al.* 2010), thereby exerting an important effect on plant defense (Jassbi *et al.* 2008).

1.6.2. Conifer-feeding beetles: the pine weevil and bark beetles

The pine weevil

The pine weevil, *Hylobius abietis* (Coleptera: Curculionidae: Molytinae) is a major pest of managed conifer forests in Europe (Leather *et al.* 1999; Nordlander *et al.* 2011). It feeds on the bark and cambium of different conifer species (particularly Scots pine, *Pinus sylvestris*, and Norway spruce, *Picea abies*), causing over 80% mortality of newly planted conifer seedlings (Pettersson and Orlander 2003). Due to its economic importance, the biology and ecology of the pine weevil, as well as the effect this species exerts on conifers as herbivores, have been extensively studied in recent years (Wallertz *et al.* 2006; Wainhouse *et al.* 2014).

In spring, after hibernation in the soil, adult pine weevils disperse by flight searching for appropriate sites to reproduce (Solbreck 1980). In managed forests weevils are attracted to volatiles released from fresh conifer stumps (Solbreck and Gyldberg 1979). During summer, and after a feeding and mating period, females oviposit in the bark of stump roots or in the surrounding soil (Nordlander *et al.* 1997). Upon hatching, larvae feed and tunnel in the bark of the stump roots where they go through four larval stages before pupation (Leather *et al.* 1999). After emergence, adults feed on conifer bark both above and below ground (Nordlander *et al.* 2005; Wallertz *et al.* 2006). Thus, the pine weevil is associated throughout its life cycle with conifer tissues, and encounters high concentrations of terpenoids. As these compounds are known to be toxic to many insects (Keeling and Bohlmann 2006), the pine weevil must have evolved strategies to detoxify or cope with them. It remains unclear though, whether they do this on their own or in association with microorganisms.

To date, characterization of the gut bacterial community of the pine weevil has been limited. Connord and colleagues (2008) described an intracellular rod shaped bacterium in the ovariole and gut crypts of both *H. abietis* and *H. transversovittatus.* This endosymbiont is closely related to *Candidatus* Nardonella, the primary symbiont of Dryopthoridae weevils (Lefevre *et al.* 2004). Although the role of this pine weevil endosymbiont has not been functionally characterized, it has been proposed to aid in the exploitation of conifers as a food source by supplementing the diet with essential nutrients (Conord *et al.* 2008), such as amino acids or vitamins (Kuriwada *et al.* 2010).

Bark beetles

Bark beetles (Coleoptera, Curculionidae, Scolytinae), which feed mostly on woody plants, are one of the most abundant insect groups with around 6000 described species (Knízek and Beaver 2004). They have been extensively studied given their economic impact and high diversity in feeding habits (Six and Klepzig 2004). Bark beetles build galleries within tree's phloem, where oviposit and their larvae feed and develop. Although most species live on weak or killed trees, some can kill their host tree or live in healthy ones (Six and Wingfield 2011).

Among conifer-feeding bark beetles, the genus *Dendroctonus* with 19 species, is one of the most comprehensively studied (Six and Klepzig 2004). Within this genus, bark beetles have been classified in three groups according to their host use, which many influence their associated microbial communities (Six and Klepzig 2004, Six 2012): (i) aggressive bark beetles that kill trees (*D. frontalis, D. ponderosae*); (ii) parasite beetles that do not kill trees (*D. micans, D. punctatus*); and (iii) successional saprophages

that feed on dead trees (*D. approximatus*). The well-studied *D. valens* uses hosts flexibly and can be classified as a saprophage as well as parasite (Smith 1971, Lindgren and Raffa 2013, Sun *et al.* 2013).

In contrast to the pine weevil, extensive research has been carried out regarding both fungal as well as bacterial symbiotic communities in bark beetles, among which filamentous fungi are the best studied (Six and Paine 1998; Ayres *et al.* 2000; Bleiker and Six 2007). Aggressive bark beetles are often dependent on symbiotic fungi for nutrition, given that they supplement their host diet with nitrogen and sterols (Ayres *et al.* 2000). It has also been proposed that symbiotic fungi might contribute to the degradation of conifer chemical defenses (Six and Klepzig 2004), although there is some controversy (Six and Wingfield 2011). On the other hand, parasitic bark beetles seem not be associated with mutualistic fungi (Six and Bracewell 2015). Yeasts are also common associates of bark beetles, especially aggressive ones, where they provide fitness benefits to their host through their involvement in pheromone communication, suppressing competitors, and enhancing growth (Zhao *et al.* 2015, Davies *et al.* 2013, Adams *et al.* 2008). Symbiotic bacterial communities in bark beetles have been hypothesized to play a nutritional role by supplementing nutrients (Morales Jimenez 2013) or detoxifying conifer chemical defenses (Adams *et al.* 2013, Boone *et al.* 2013), although the latter has not been demonstrated in vivo.

1.7. THESIS OUTLINE

As presented above, my interests lie on the adaptive nature of several traits involved in plant-insect interactions, in particular plant defenses, insect counter adaptations and symbiosis. Accordingly, i have explored the possibility that conifer-feeding beetles associate with microbial organisms in order to overcome plant defenses.

In this doctoral project I have investigated (1) the effect of conifer terpenoid defenses on herbivores; (2) whether the gut community of conifer-feeding beetles is conserved across geographical distances and species, specifically focusing on pine weevils and bark beetles; and (3) whether this bacterial assembly can mediate the degradation of host plant defenses and its effect on host fitness. I have also investigated (4) the possibility of exploiting existing and developing knowledge on insect-microbe associations for application in biotechnology.

In chapter 2, an isoprenyl diphosphate synthase was overexpressed to study its role in terpene synthesis, thereby altering terpenoid phenotypes in Norway spruce. I assessed the role these different chemical profiles have in plant defense against herbivores.

In chapter 3 I characterized the microbial community of the pine weevil (*Hylobius abietis*) in order to assess its overall geographical stability. I also compared this microbial assembly to that of other beetles exploiting similar and different ecological niches, respectively, to gain insights into the adaptive nature of this community assembly. We also searched the microbial community for potential terpene-degrading bacteria by *in silico* approaches.

In chapter 4 the fungal and bacterial community of several species of *Dendroctonus* beetles with different host plant use were characterized and compared to the microbiota of their surrounding habitat.

In chapter 5 I explored whether the microbiota of the pine weevil is able to mediate the degradation of its host plant secondary metabolites *in vitro* and *in vivo*, and whether this could represent an adaptive strategy to overcome coniferous plant defenses.

In chapter 6 my coauthors and I discuss the possibility of using our knowledge on symbiosis to control pest species or vector-borne diseases. We argue that we can also exploit this knowledge in order to perform targeted searches when looking for specific enzymatic functions and metabolites that are interesting for biotechnological purposes.

Finally, in chapter 7 I discuss these findings integrating them in what is already known in the field of symbiosis.

1.8. REFERENCES

Adams AS, Six DL, Adams SM, Holben WE (2008) In vitro interactions between yeasts and bacteria and the fungal symbionts of the mountain pine beetle (*Dendroctonus ponderosae*). *Microb Ecol* 56:460–466.

Adams AS, Aylward FO, Adams SM et al (2013) Mountain pine beetles colonizing historical and naive host trees are associated with a bacterial community highly enriched in genes contributing to terpene metabolism. *Appl Environ Microbiol* 79:3468–3475.

Agrawal AA, Konno K (2009). Latex: a model for understanding mechanisms, ecology and evolution of plant defense against herbivory. *Annu. Rev. Ecol. Evol*. 40: 311-331.

Agrawal AA, Petschenka G, Bingham RA, Weber MG, Rasmann S (2012). Toxic cardenolides: chemical ecology and coevolution of specialized plant-herbivore interactions. *New Phytologist*. 194: 28-45.

Ahn, S.J., Vogel, H., Heckel, D.G., 2012. Comparative analysis of the UDP-glycosyltransferase multigene family in insects. *Insect Biochem. Mol. Biol*. 42, 133-147.

Ayres MP, Wilkens RT, Ruel JJ, Lombardero MJ, Vallery E (2000) Nitrogen budgets of phloem-feeding bark beetles with and without symbiotic fungi. *Ecology*, 81, 2198–2210.

Banyai, W., Kirdmanee, C., Mii, M., Supaibulwatana, K. Overexpression of farnesyl pyrophosphate synthase (*FPS*) gene affected artemisinin content and growth of *Artemisia annua* L. *Plant Cell Tiss Organ Cult* (2010) 103: 255.

Barr KL, Hearne LB, Briesacher S, Clark TL, Davis GE (2010) Microbial symbionts in insects influence down-regulation of defense genes in maize. PLoS ONE 5(6): e11339. doi:10.1371/journal.pone.0011339

Becerra XJ. (1994) Squirt-gun defense in Bursera and the chrysomelid counterploy. *Ecol. Soc. Am.* 75: 1991-1996.

Bede JC. et al. (2006) Caterpillar herbivory and salivary enzymes decrease transcript levels of *Medicago truncatula* genes encoding early enzymes in terpenoid biosynthesis. *Plant Mol. Biol.* 60, 519–531.

Bellés X, Martín D, Piulachs MD (2005). The mevalonate pathway and the synthesis of juvenile hormone in insects. *Ann. Rev. Ent* 50, 181-199.

Bennet G, Moran NA (2015). Heritable symbiosis: the advantages and perils of an evolutionary rabbit hole. *Proc. Natl. Acad. Sci. USA*. 112 (33) 10169-10176.

Berenbaum MR (1986). Target site insensitivity in insect-plant interactions. In "Molecular aspects of insect-plant associations". Plenum Press, New York. pp-257-272.

Bittleston LS, Pierce NE, Ellison AM, Pringle A (2016). Convergence in multispecies interactions. *Trends in Ecol. Evol.* 2053.

Bleiker, K.P., Six D. (2007). Dietary benefits of fungal associates to an eruptive herbivore: Potential implications of multiple associates on host population dynamics. *Environ. Entomol.* 36(6):1384 –1396.

Boll M, Löffler C, Morris BEL, Kung JW (2014) Anaerobic degradation of homocyclic aromatic compounds via arylcarboxyl-coenzyme A esters: organisms, strategies and key enzymes. *Environ Microbiol* 16:612–627

Boone CK, Keefover-Ring K, Mapes AC et al. (2013) Bacteria associated with a tree-killing insect reduce concentrations of plant defense compounds. *Journal of Chemical Ecology*, 39, 1003–1006.

Carroll M, Hanlon A, Hanlon T, Zangerl AR, Berenbaum MR (1997) Behavioral effects of carotenoid sequestration by the parsnip webworm, *Depressaria pastinacella. J. Chem. Ecol. 23*, 2707–2719

Cazzonelli CI and Pogson BJ (2010). Source to sink: regulation of carotenoid biosynthesis in plants. *Trends Plant Sci*. 15:266–274

Ceja-Navarro JA, Vega FE, Karaoz U, Hao Z, Jenkins S, Lim HC, Kosina P, Infante F, Northen TR, Brodie EL. (2015) Gut microbiota mediate caffeine detoxification in the primary insect pest of coffee. *Nat. Comm.* **6**, 7618.

Chung SH, Rosa C, Scully ED, Peiffer M, Tooker JF, Hoover K, Luthe DS, Felton GW (2013). Hervivore exploits orally secreted bacteria to suppress plant defenses. *Proc. Nat. Acad. Sci. USA* 110, 39: 15728-15733.

Clayton TA, Baker D, Lindon JC et al (2009) Pharmacometabonomic identification of a significant host-microbiome metabolic inter- action affecting human drug metabolism. *Proc Natl Acad Sci USA* 106:14728–14733

Conord C, Despres L, Vallier A, Balmand S, Miquel C, Zundel S, Lemperiere G, Heddi A (2008). Long-term evolutionary stability of bacterial endosymbiosis in Curculionoidea: additional evidence of symbiont replacement in the Dryophtoridae family. *Mol. Biol. Evol.* 25, 5: 859-868.

Consales F, Schweizer F, Erb M, Gouhier-Darimont C, Bodenhausen N, Bruessow F, Sobhy I, Reymond P (2012). Insect oral secretions suppress wound-induced responses in *Arabidopsis*. *J. Exp. Bot*. 63(2): 727-737.

David LA, Maurice CF, Carmody RN et al (2014) Diet rapidly and reproducibly alters the human gut microbiome. *Nature* 505:559–563.

Davis TS, Crippen TL, Hofstetter RW et al. (2013). Microbial volatile emissions as insect semiochemicals. *J. Chem. Ecol*. 39, 840-859.

DeFine Licht H, Schiott M, Rogowska-Wrzesinska A, Nygaard S, Roepstorff P, Boomsma JJ (2013). Laccase detoxification mediates the nutritional alliance between leaf-cutting ants and fungus-garden symbionts. *Proc. Natl. Acad. Sci. USA* 110, 583-587.

De Geyter E, Swevers L, Soin t, Geelen D, Smagghe G (2012). Saponins do not affect the ecdysteroid receptor complex but cause membrane permeation in insect culture cells. *J. Insect. Physiol* 58(1): 18-23.

Delphia, CM., Mescher, MC., De Moraes, CM. (2007) Induction of plant volatiles by herbivores with different feeding habits and the effects of induced defenses on host-plant selection by thrips. *J. Chem. Ecol.* 33: 997-1012.

Despres L, David JP, Gallet C (2007). The evolutionary ecology of insect resistance to plant chemicals. *Trends. Ecol. Evol.* 22, 6: 298-307.

Dewick PM (2002). The biosynthesis of C5-C25 terpenoid compounds. *Roy. Soc. Chem*. 19, 181-222.

Discher S, Burse A, Tolzin-Banasch, Heinemann SH, Pasteels JM, Boland W (2009). A versatile transport network for sequestering and excreting plant glycosides in leaf beetles provides an evolutionary flexible defense strategy.

Dobler, S., Dalla, S., Wagschal, V., Agrawal, A. (2012) Community-wide convergent evolution in insect adaptation to toxic cardenolides by substitutions in the NA,K-ATPase. *Proc. Natl. Acad. Sci. USA*. 109: 13040-13045.

Douglas AE (2009). The microbial dimension in insect nutritional ecology. *Funct. Ecol* 23(1): 38-47.

Dufourc EJ (2008). Sterols and membrane dynamics J. Chem. Biol 1: 1-4.

Dussourd DE. 1993. Foraging with finesse caterpillar adaptations for circumventing plant defenses. In Caterpillars: Ecological and Evolutionary Constraints on Foraging, ed. NE Stamp, TM Casey, pp. 92–131. New York/London: Chapman & Hall

Dussourd DE, Denno RF. 1994. Host range of generalist caterpillars: trenching permits feeding on plants with secretory canals. Ecology 75:69–78

Edger et al. (2015) The butterfly plant arms-race escalated by gene and genome duplication. *Proc. Nat. Ac. Sciences USA* 112 (27) 8362-8366.

Enayati, A.A. et al. (2005) Insect glutathione transferases and insecticide resistance. *Insect Mol. Biol*. 14, 3–8

Erb M and Robert CAM (2016). Sequestration of plant secondary metabolites by insect herbivores: molecular mechanisms and ecological consequences. *Current Opinion in Insect Science*. 14, 8-11.

Farjon A and Page CN (compilers) (1999). Global assessment of conifer diversity and threats. In "Conifers. Status survey and conservation action plan". IUCN/SSC Conifer Specialist Group. IUCN, Gland, Switzerland and Cambridge, UK. Pp 2-6.

Farrell B., Dussourd, D., Mitter, M. (1991) Escalation of plant defense: do latex and resin canals spur plant diversification? *The American Naturalist* 138, 4: 881-900.

Fernandes GW (1994). Plant mechanical defenses against insect herbivory. *Revista Brasileira de Entomologia*. **38** (2): 421–433

Feyereisen, R. (2005) Insect cytochrome P450. In Comprehensive Molecular Insect Science (Gilbert, L.I. et al., eds), pp. 1–77, Elsevier.

Franceschi VR, Krokene P, Christiansen E, Krekling T (2005). Anatomical and chemical defenses of conifer bark against bark beetles and other pests. *New Phytol*. 167 (2) 353-75.

Franche, C, Lindström, K, Elmerich, C. (2009). Nitrogen-fixing bacteria associated with leguminous and non-leguminous plants. *Plant and Soil* 321:35.

Francis F, Vanhaelen N, Haubruge E (2005). Glutathione S-transferases in the adaptation to plant secondary metabolites in the *Myzus persicae* aphid. *Arch. Insect Biochem. Physiol.* 58(3): 166-174.

Futuyma D (2000) Some current approaches to the evolution of plant-herbivore interactions. *Plant Spec. Biol*. 15: 1-9.

Fürstenberg-Hägg J, Zagrobelny M, Bak S (2013). Plant defense against insect herbivores. *Int. J. Mol. Sci.* 14 (5)): 10242-10297.

Garcia-Amado MA, Michelangeli F, Gueneau P, Perez ME. (2007). Bacterial detoxification of saponins in the crop of the avian foregut fermenter *Opisthocomus hoazin*. *J Ani Feed Sci* **16**: 82–85.

Ghershenzon J, Dudareva N (2007). The function of terpene natural products in the natural world. *Nat. Chem. Biol*. 3, 7: 408-414.

Hammer TJ, Bowers MD (2015). Gut microbes may facilitate insect herbivory of chemically defended plants. *Oecologia*. 179: 1-14.

Hammerbacher A, Schmidt A, Wadke N, Wright LP, Schneider B, Bohlmann J, Brand WA, Fenning TM, Gershenzon J (2013). A common fungal associate of the spruce bark beetle metabolizes the stilbene defenses of Norway spruce. *Plant Phys*. 162, 3: 1324-1336.

Hehemann J-H, Kelly AG, Pudlo NA et al (2012) Bacteria of the human gut microbiome catabolize red seaweed glycans with carbohydrate-active enzyme updates from extrinsic microbes. *Proc Natl Acad Sci* USA 109:19786–19791

Hemmerlin A, Harwood JL, Bach TJ (2012). A raison d'êttre for two distinct pathways in the early steps of plant isoprenoid biosynthesis? Progr. Lipid Res. 51, 2:95-148.

Henry, R.J.(2005) Plant Diversity and evolution. London: CABI.

Hopkins RJ, van Dam NM, van Loon JJA (2009). Role of glucosinolates in insect-plant relationships and multitrophic interactions. *Ann. Rev. Entomol*. 54: 57-83.

Huber DPW, Bohlman J (2006). The role of terpene synthases in the direct and indirect defense of conifers against insect herbivory and fungal pathogens. In "Multigenic and Induced systemic resistance in plants". Pp 296-313.

Jassbi, AR, Gase K, Hettenhausen C, Schmidt A, and Baldwin IT (2008). Silencing geranylgeranyl diphosphate synthase in *Nicotiana attenuata* dramatically impairs resistance to *Manduca sexta. Plant Physiol*. 146 974–986

Jeschke V, Gershenzon J, Giddings Vassao D (2015). Metabolism of glucosinoltes and their hydrolysis products in insect herbivores. In: Jetter R (ed). The formation, structure and activity of phytochemicals, recent advances in phythochemistry 45.

Kant MR, Ament K, Sabelis MW, Haring MY, Schuurink RC (2004). Differential timing of spider mite-induced direct and indirect defenses in tomato plants. *Plant Phys*. 135: 483-495.

Kaplan I, Halitschke R, Kessler A, Sardanelli S, Denno RF (2008). Constitutive and induced defenses to herbivory in above- and belowground plant tissues. *Ecology* 89(2): 392-406.

Karban R, Baldwin IT (1997) Induced Responses to Herbivory. University of Chicago Press, Chicago.

Keeling CI, Bohlmann J (2006). Genes, enzymes and chemicals of terpenoid diversity in the constitutive and induced defence of conifers against insects and pathogens. *New Phytologist* 170: 657-675.

Kikuchi Y, Hayatsu M, Hosokawa T, Nagayama A, Tago K, Fukatsu T (2012). Symbiont-mediated insecticide resistance. *Proc. Natl. Acad. Sci. USA* 109, 22: 8618-8622.

Knízek M, Beaver R (2004). Taxonomy and systematics of bark and ambrosia beetles. In: Lieutier F, Day KR, Battisti A et al. (eds) Bark and wood boring insects in living trees in Europe, a synthesis. Kluwer, Dordrecht, pp 41-54.

Kohl KD, Connelly JW, Dearing MD, Forbey JS (2016) Microbial detoxification in the gut of a specialist avian herbivore, the Greater Sage-Grouse. *FEMS Microbiology Letters*. 363: fnw144.

Kohl, K.D., Dearing, M.D. (2012) Experience matters: prior exposure to plant toxins enhances diversity of gut microbes in herbivores. *Ecology Letters*. 15: 1008-1015.

Kohl KD, Weiss RB, Cox J et al (2014) Gut microbes of mammalian herbivores facilitate intake of plant toxins. *Ecol Lett* 17:1238–1246

Konno K, Hirayama C, Shinbo H (1997). Glycine in digestive juice: a strategy of herbivorous insects against chemical defense of host plants*. J. Insect Phys*. 43, 3: 217-224.

Konno K, Yasui H, Hirayama C, Shinbo H (1998) Glycine protects against strong protein-denaturing activity of oleuropein, a phenolic compound in privet leaves. *J. Chem. Ecol*. 24, 4: 735-751.

Kölsch G, Matz-Grund C & Pedersen BV (2009) Ultrastructural and molecular characterization of endosymbionts of the reed beetle genus *Macroplea* (Chrysomelidae, Donaciinae), and proposal of "Candidatus *Macropleicola* appendiculatae" and "Candidatus *Macropleicola* muticae". *Can J Microbiol* 55: 1250–1260.

Kuhn, J., Pettersson, EM., Feld, BK., Burse, A., Termnoia, A., Pasteels, JM., Boland, W. (2004). Selective transport systems mediate sequestration of plant glucosidases in leaf beetles: a molecular basis for adaptation and evolution. *Proc. Natl. Acad. Sci. USA.* 101: 13808-13813.

Kuriwada T, Hosokawa T, Kumano N, Shiromoto K, Haraguchi D, Fukatsu T (2010) Biological role of *Nardonella* endosymbiont in its weevil host. *PLoS ONE* 5(10): e13101. doi:10.1371/journal.pone.0013101.

Labandeira CC, Currano ED (2013). The fossil record of plant-insect dynamics. *Earth and Planetary Sciences* 41: 287-311.

Lagenheim JH (2003). Plant resins: chemistry, evolution, ecology and ethnobotany. Portland, Cambridge: Timber Press.

Laskin, AI, Lechevalier HA (1984). CRC Handbook of Microbiology. 2nd edn., vol. 5. CRC Press, Boca Raton FL.

Lauzon CR, Potter SE, Prokopy RJ. (2003). Degradation and detoxification of dihydrochalcone Phlorizin by Enterobacter agglomerans, a bacterium associated with the apple pest, Rhagoletis pomonella (Walsh) (Diptersa: Tephritidae). *Environ Entomol* 32: 953–962.

Leather S, Day KR, Salisbury AN (1999). The biology and ecology of the large pine weevil*, Hylobius abietis* (Coleoptera: Curculionidae): a problem of dispersal? *Bull Entomol Res* 89: 3–16.

Lefèvre C, Charles H, Vallier A, Delobel B, Farrel B, Heddi A (2004). Endosymbiont phylogenesis in the dryopthoridae weevils: evidence for bacterial replacement. Mol. Biol. Evol. 21, 6: 965-973.

Letourneau D (1988) Microorganisms as mediators of intertrophic and intratrophic interactions. In: Barbosa P, Letourneau D (eds) Novel aspects of insect–plant interactions. Wiley, pp 91–95

Lindgren BS, Raffa KF (2013). Evolution of tree killing in bark beetles. *Can. Entomol*. 145: 471-495.

Litvak ME, Monson RK (1998). Patterns of induced and constitutive monoterpene production in conifer needles in relation to insect herbivory. *Oecologia* 114: 531-540

Login FH, Balmand S, Vallier A, Vincent-Monegat, Vigneron A, Weiss-Gayet M, Rochat D, Heddi A (2011). Antimicrobial peptides keep insect endosymbionts under control. *Science* 334: 362-365.

Lombard J, Moreirra D (2011). Origins and early evolution of the mevalonate pathway of isoprenoid biosynthesis in the three domains of life. *Mol. Biol. Evol*. 28, 1: 87-99.

Lucas PW, Turner IM, Dominy NJ, Yamashita N (2000). Mechanical defences to herbivory. *Annals of Botany* 86: 913-920

Martin D, Tholl D, Gershenzon J, Bohlmann J (2002) Methyl jasmonate induces traumatic resin ducts, terpenoid resin biosynthesis, and terpenoid accumulation in developing xylem of Norway spruce stems. *Plant Physiol* 129: 1003–1018.

Martin D, Fäldt J, Bohlmann J (2004) Functional characterization of nine Norway spruce TPS genes and evolution of gymnosperm terpene synthases of the TPS-d subfamily. *Plant Physiol* 135 1908–1927

Mason CJ, Couture JJ, Raffa KF (2014). Plant-associated bacteria degrade defense chemicals and reduce their adverse effects on an insect defoliator. *Oecologia* 175:901–910.

McCullough DC, Wagner MR (1993) Defusing host defenses: ovipositional adaptations of sawflies to plant resins. *Academic Press, San Diego*.

McCutcheon, JP., McDonald, BR., Moran, NA. (2009). Convergent evolution of metabolic roles in bacterial co-symbionts of insects. *Proc. Natl. Acad. Sci. USA.* 106: 15394-15399.

McFall Ngai M, Hadfield MG, Bosch TCG, Carey HV, Domazet-Loso T, Douglas AE, Dubilier N, Eberl G, Fukami T, Gilbert SFm Hentschel U, King N, Kjelleberg S, Knoll AH, Kremer N, Mazmanian SK, Metcalf JL, Nealson K, Pierce NE, Rawls JF, Reid A, Rubi EG, Rumpho M, Sanders JG, Tautz D, Wernegreen JJ (2013) Animals in a bacterial world. *Proc. Nat. Acad. Sci. USA* 110(9) 3229-3236.

Miller AW, Kohl KD, Denise Dearing M (2014) The gastrointestinal tract of the white-throated woodrat (*Neotoma albigula*) harbors distinct consortia of oxalate-degrading bacteria. *Appl Environ Microbiol* 80:1595–1601

Mithoefer A, Boland W (2012). Plant defense against herbivores: chemical aspects. *Annu. Rev. Plant Biol*. 63: 431-450.

Morales-Jimenez J, Vera-Ponce de León A, García-Domínguez A, Martínez-Romero E, Zúñiga G, Hernández-Rodríguez C (2013). Nitrogen-fixing and urolytic bacteria associated with the gut of *Dendroctonus rhizophagous* and *Dendroctonus valens* (Curculiondae: Scolytinae). *Microb. Ecol.* 66, 200-210.

Moran N. (2007) Symbiosis as an adaptive process and source of phenotypic complexity. *Proc. Natl. Acad. Sci. USA.* 104: 8627-8633.

Mulkidjanian, AY., Koonin, EV., Makarova, KS., Mekhedov, SL., Sorokin, A., Wolf, YI., Dufresne, A., Partnesky, F., Burd, H., Kaznadzey, D., Haselkorn, R., Galperin, MY. (2006). The cyanobacterial genome core and the origin of photosynthesis. *Proc. Natl. Acad. Sci. USA.* 103: 13126-13131.

Mullin C (1986) Adaptive divergence of chewing and sucking arthro- pods to plant allelochemicals. In: Brattsten LB, Ahmad S (eds) Molecular aspects on insect-plant associations. Springer, Berlin Heidelberg New York pp 175–209

Musser, R.O. et al. (2002) Herbivory: Caterpillar saliva beats plant defences - a new weapon emerges in the evolutionary arms race between plants and herbivores. *Nature* 416, 599–600.

Nagel, R., Berasategui, A., Paetz, C., Gershenzon, J., Schmidt, A. (2014) Overexpression of an isoprenyl diphosphate synthase in spruce leads to unexpected terpene diversion products that function in plant defense. *Plant Physiology* Vol. 164, pp. 555–569

Nathanson JA (1984). Caffeine and related methylxanthines: possible naturally occurring pesticides. *Science* 226, 184-187

Nealis, V.G. and Nault, J.R. (2005) Seasonal changes in foliar terpenes indicate suitability of Douglas-fir buds for western spruce budworm. *J. Chem. Ecol*. 31, 683–696

Nordlander G, Nordenhem H, Bylund H (1997) Oviposition patterns of the pine weevil *Hylobius abietis*. *Entomologia Experimentalis et Applicata*, 85, 1–9.

Nordlander G, Bylund H, Bjorklund N (2005) Soil type and microtopography influencing feeding above and below ground by the pine weevil *Hylobius abietis*. *Agricultural and Forest Entomology*, 7, 107–113.

Nordlander G, Hellqvist C, Johansson K, Nordenhem H (2011) Regeneration of European boreal forests: effectiveness of measures against seedling mortality caused by the pine weevil *Hylobius abietis. Forest Ecology and Management*, 262, 2354–2363.

Ode, PJ (2006) Plant chemistry and natural enemy fitness: effects on herbivore and natural enemy interactions. *Annu. Rev. Entomol*. 51, 163–185

Ohnuma S, Hirooka K, Tsuruoka N, Yano M, Ohto C, Nakane H, Nishino T (1998) A pathway where polyprenyl diphosphate elongates in prenyltransferase: insight into a common mechanism of chain length determination of prenyltransferases. *J Biol Chem* 273: 26705–26713

Parr JC, Thurston R (1972) Toxicity of nicotine in synthetic diets to larvae of the tobacco hornworm. *Ann Entomol Soc Am* 65:1185–1188

Payne SH, Loomis WF (2006). Retention and loss of amino acid biosynthetic pathways based on analysis of whole-genome sequences. *Eukaryot Cell* 5(2): 272-276.

Petersson M, Orlander G (2003) Effectiveness of combinations of shelterwood, scarification, and feeding barriers to reduce pine weevil damage. *Canadian Journal of Forest Research – Revue Canadienne De Recherche Forestiere*, 33, 64–73.

Petschenka G and Agrawal A (2016). How herbivores coopt plant defenses: natural selection, specialization, and sequestration. *Current Opinion in Insect Science*. 14, 17-24.

Petschenka G, Fandrich S, Sander N, Wagschal V, Boppré M, Dobler S (2013). Stepwise evolution of resistance to toxic cardenolides via genetic substitutions in the Na+/K+ -ATPase of milkweed butterflies (Lepidoptera: Danaini). *Evolution* 67(9): 2753-61.

Pentzhold S, Zagrobelny M, Rook F, Bak S (2014). How insects overcome two-component plant chemical defence: plant ß-glucosidases as the main target for herbivore adaptation. *Biol. Rev*. 89, 531-551.

Phillips MA, Croteau RB (1999). Resin-based defenses in conifers. *Trends in Plant Science* 4 (5) 184-190.

Pichersky E and Lewinsohn E (2011). Convergent evolution in plant specialized metabolism. *Annu. Rev. Plant. Biol.* 62: 549-566.

Richards LA, Lampert EC, Bowers MD et al (2012) Synergistic effects of iridoid glycosides on the survival, development and immune response of a specialist caterpillar, *Junonia coenia* (Nymphalidae*). J Chem Ecol* 38:1276–1284

Robert CAM, Frank DL, Leach KA, Turlings TCJ, Hibbard BE, Erb M (2013). Direct and indirect plant defenses are not suppressed by endosymbionts of a specialist root herbivore. *J. Chem. Ecol*. 39: 507-515.

Roskov Y, Abucay L, Orrell T, Nicolson D, Flann C, Bailly N, Kirk P, Bourgoin T, DeWalt RE, Decock W, De Wever A, eds. (2016). Species 2000 & ITIS Catalogue of Life, 2016 Annual Checklist. Digital resource at www.catalogueoflife.org/annual-checklist/2016. Species 2000: Naturalis, Leiden, the Netherlands. ISSN 2405-884X.

Salem H, Florez L, Gerardo N, Kaltenpoth M (2015). An out-of-body experience: the extracellular dimension for the transmission of mutualistic bacteria in insects. *Proc. Roy. Soc.* B 282: 20142957.

Schmidt A, Nagel R, Krekling T, Christiansen E, Gershenzon J, Krokene P (2011). Induction of isoprenyl diphosphate synthases, plant hormones and defense signaling genes correlates with traumatic resin duct formation in Norway spruce (*Picea abies*). *Plant Molecular Biology*, 77(6), 577-590.

Schmidt, A., Gershenzon, J. (2007). Cloning and characterization of isoprenyl diphosphate synthases with farnesyl diphosphate and geranylgeranyl diphosphate synthase activity from Norway spruce (*Picea abies*) and their relation to induced oleoresin formation. *Phytochemistry*, 68(21), 2649-2659.

Schmidt, A., Gershenzon, J. (2008). Cloning and characterization of two different types of geranyl diphosphate synthases from Norway spruce (*Picea abies*). *Phytochemistry*, 69(1), 49-57.

Shao Y., Spiteller D., Tang X., Ping L., Colesie C., Münchberg U., et al. (2011). Crystallization of α- and β-carotene in the foregut of *Spodoptera* larvae feeding on a toxic food plant. *Insect Biochem. Mol. Biol*. 41 273–281.

Shen SK, Dowd PE (1990). Insect symbionts: A promising source of detoxifying enzymes. In: Agricultural and Synthetic Polymers: Biodegradability and Utilization. J. E. Glass and G. Swift, Eds. American Chemical Society. Washington. D.C. pp. 33-37.

Six, D.L. 2012. Ecological and Evolutionary determinants of bark beetle-fungus symbioses. *Insects* 3: 339-366.

Six DL, Bracewell R (2015). *Dendroctonus*. In: Vega FE, Hofstetter RW (ed.). Bark Beetles. San Diego: Academic Press, 305-50.

Six DL, Klepzig K (2004). *Dendroctonus* bark beetles as model systems for studies on symbiosis. *Symbiosis* 37: 207-232.

Six, D.L., Paine TD. (1998). Effects of mycangial fungi and host tree species on progeny survival and emergence of *Dendroctonus ponderosae* (Coleoptera: Scolytidae). *Environ. Entomol.* 27(6):1393–1401.

Six DL, Wingfield MJ (2011). The role of phytopathogenicity in bark beetle-fungus symbioses: a challenge to the classic paradigm. *Annu. Rev. Entomol*. 56, 255-272.

Smith RH (1971). Red turpentine beetle. US Department of Agriculture, Forest Service, Forest Pest Leaflet, 1-9.

Solbreck C (1980) Dispersal distances of migrating pine weevils, *Hylobius abietis*, Coleoptera – Curculionidae. *Entomologia Experimentalis et Applicata*, 28, 123–131.

Solbreck C, Gyldberg B (1979) Temporal flight pattern of the large pine weevil, *Hylobius abietis* L (Coleoptera, Curculionidae), with special reference to the influence of weather. *Zeitschrift Fur Angewandte Entomologie – Journal of Applied Entomology*, 88, 532–536.

Stotz HU, Pittendrigh BR, Kroymann J, Weniger K, Fritsche J, Bauke A, Mitchell-Olds T (2000). Induced plant defense responses against chewing insects. Ethylene signaling reduces resistance of *Arabidopsis* against Egyptian cotton worm but not Diamondback moth. *Plant Phys* 124, 1007-117

Strauss AS, Peters S, Boland W and Burse A (2013). ABC transporter functions as a pacemaker for sequestration of plant glucosides in leaf beetles. Elife 2, p. e01096

Sun J, Lu M, Gillette NE et al. (2013). Red turpentine beetle: innocuous native becomes invasive tree killer in China. *Annu. Rev. Entomol*. 58, 293-311.

Toju H, Tanabe AS, Notsu Y, Sota T, Fukatsu T (2013). Diversification of endosymbiosis: replacements, co-speciation and promiscuity of bacteriocyte symbionts in weevils. The ISME Journal 7, 1378-1390.

Trapp SC, Croteau RB (2001). Genomic organization of plant terpene synthases and molecular evolutionary implications. Genetics 158, 2: 811-832.

Vigneron A, Masson F, Vallier A, Balmand S, Rey M, Vincent-Monegat C, Aksoy E, Aubailly-Giraud E, Zaidman-Remy A, Heddi A (2014). Insects recycle endosymbionts when the benefit is over. *Curr. Biol.* 24(19): 2267-2273.

Wainhouse D, Inward DJG, Morgan G (2014) Modelling geographical variation in voltinism of *Hylobius abietis* under climate change and implications for management. *Agricultural and Forest Entomology*, 16, 136–146.

Wallertz K, Nordlander G, Orlander G (2006) Feeding on roots in the humus layer by adult pine weevil, Hylobius abietis. *Agricultural and Forest Entomology*, 8, 273–279.

Weber MG (2014) Defense mutualisms enhance plant diversification. *Proc. Natl. Ac. Sci*. 111: 16442-16447.

Welte CU, de Graaf RM, van den Bosch TJM, Op den Camp HJ, van Dam NM, Jetten MSM. 2016 Plasmids from the gut microbiome of cabbage root fly larvae encode SaxA that catalyzes the conversion of the plant toxin 2-phenylethyl isothiocyanate. *Environ. Microbiol*. 18 1379-90.

Whiteman, NK., Mooney, KA. (2012) Evolutionary biology: insects converge on resistance. *Nature* 7416: 376-377.

Whitaker MRL, Salzman S, Sanders J, Kaltenpoth M, Pierce NE (2016). Microbial communities of lycaenid butterflies do not correlate with larval diet. *Front. Microbiol.* 7: 1920.

Wittstock, U., Gershenzon, J. (2002) Constitutive plant toxins and their role in defense against herbivores and pathogens. *Curr. Opin. Plant Biol.* 5: 300-307.

Yang, Z. et al. (2005) Molecular dynamics of detoxification and toxin- tolerance genes in brown plant hopper (*Nilaparvata lugens* Stal., Homoptera: Delphacidae) feeding on resistant rice plants. *Arch. Insect* Biochem. *Physiol.* 59, 59–66

Yanovsky C (2007). RNA-based regulation of genes of tryptophan synthesis and degradation, in bacteria. *RNA* 13(8): 1141-1154.

Zagrobelny, M. et al. (2004) Cyanogenic glucosides and plant–insect interactions. *Phytochemistry* 65, 293–306

Zhao T, Axelsson K, Krokene P, Borg-Karlson AK (2015). Fungal symbionts of the spruce bark beetle synthetize the beetle aggregation pheromone 2-methyl-3-buten-2-ol. *J. Chem. Ecol.* 41: 848-52.

Zhu-Salzman K, Bi JL, Liu TX (2005). Molecular strategies of plant defense and insect counter-defense. *Insect Science* 12, 3-15.

CHAPTER II

OVEREXPRESSION OF AN ISOPRENYL DIPHOSPHATE SYNTHASE IN SPRUCE LEADS TO UNEXPECTED TERPENE DIVERSION PRODUCTS THAT FUNCTION IN PLANT DEFENSE

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2.1. ABSTRACT

Spruce (*Picea* spp.) and other conifers employ terpenoid-based oleoresin as part of their defense against herbivores and pathogens. The short-chain isoprenyl diphosphate synthases (IDS) are situated at critical branch points in terpene biosynthesis, producing the precursors of the different terpenoid classes. To determine the role of IDS and to create altered terpene phenotypes for assessing the defensive role of terpenoids, we overexpressed a bifunctional spruce IDS, a geranyl diphosphate and geranylgeranyl diphosphate synthase in white spruce (*Picea glauca*) saplings. While transcript level (350-fold), enzyme activity level (7-fold), and in planta geranyl diphosphate and geranylgeranyl diphosphate levels (4- to 8-fold) were significantly increased in the needles of transgenic plants, there was no increase in the major monoterpenes and diterpene acids of the resin and no change in primary isoprenoids, such as sterols, chlorophylls, and carotenoids. Instead, large amounts of geranylgeranyl fatty acid esters, known from various gymnosperm and angiosperm plant species, accumulated in needles and were shown to act defensively in reducing the performance of larvae of the nun moth (*Lymantria monacha*), a conifer pest in Eurasia. These results show the impact of overexpression of an IDS and the defensive role of an unexpected accumulation product of terpenoid biosynthesis with the potential for a broader function in plant protection.

2.2. INTRODUCTION

Terpenes are a structurally very diverse class of metabolites (Köksal *et al.* 2011) that have major roles in primary and secondary metabolism (Buchanan *et al.* 2000; Gershenzon and Dudareva, 2007). The basic pathways of terpene biosynthesis have been well studied. Two separate pathways, the mevalonate (MVA) pathway in the cytosol and peroxisomes and the methyl-erythritol phosphate (MEP) pathway in plastids, produce the universal C5 precursors for terpene biosynthesis, dimethylallyl diphosphate (DMADP) and

isopentenyl diphosphate (IDP). Next, sequential condensations of DMADP with one to three molecules of IDP yield the elongated C10, C15, and C20 intermediates, geranyl diphosphate (GDP), farnesyl diphosphate (FDP), and geranylgeranyl diphosphate (GGDP), respectively (Arigoni *et al.* 1997; Rodríguez-Concepción, 2006; Hemmerlin *et al.* 2012).

The enzymes catalyzing these condensation reactions are designated short-chain isoprenyl diphosphate synthases (IDS) and belong to the large family of prenyltransferases (Wang and Ohnuma, 2000; Liang *et al.* 2002; Fig. 1). The enzymes are named after their main products as GDP synthase, FDP synthase, and GGDP synthase and usually belong to the group of trans-IDS enzymes based on the stereochemistry of the double bond of the reaction product (Kharel and Koyama, 2003; Liang, 2009), although short chain cis-IDS enzymes are known (Sallaud *et al.* 2009; Schilmiller *et al.* 2009; Akhtar *et al.* 2013) The short-chain prenyl diphosphates produced are the substrates for terpene synthases (Chen *et al.* 2011) that form the huge variety of terpene skeletons, which in turn may be further modified, such as by cytochrome P450 oxidoreductases (Bohlmann and Keeling, 2008) or other oxidative, reductive, and conjugation processes. Thus, the IDS enzymes function at an important branch point in terpene biosynthesis where the pathway splits into routes to the major classes, such as monoterpenes (C10), sesquiterpenes (C15), diterpenes (C20), triterpenes (C30), and tetraterpenes (C40). However, little is known about how IDS control flux into the different branches. A few recent publications have addressed the importance of FDP synthases (Chen *et al.* 2000; Masferrer *et al.* 2002; Manzano *et al.* 2004, 2006; Han *et al.* 2006; Banyai *et al.* 2010; Closa *et al.* 2010; Keim *et al.* 2012), but only one focuses on a GDP synthase (Lange *et al.* 2011) and one on a GGDP synthase (Kai *et al.* 2011).

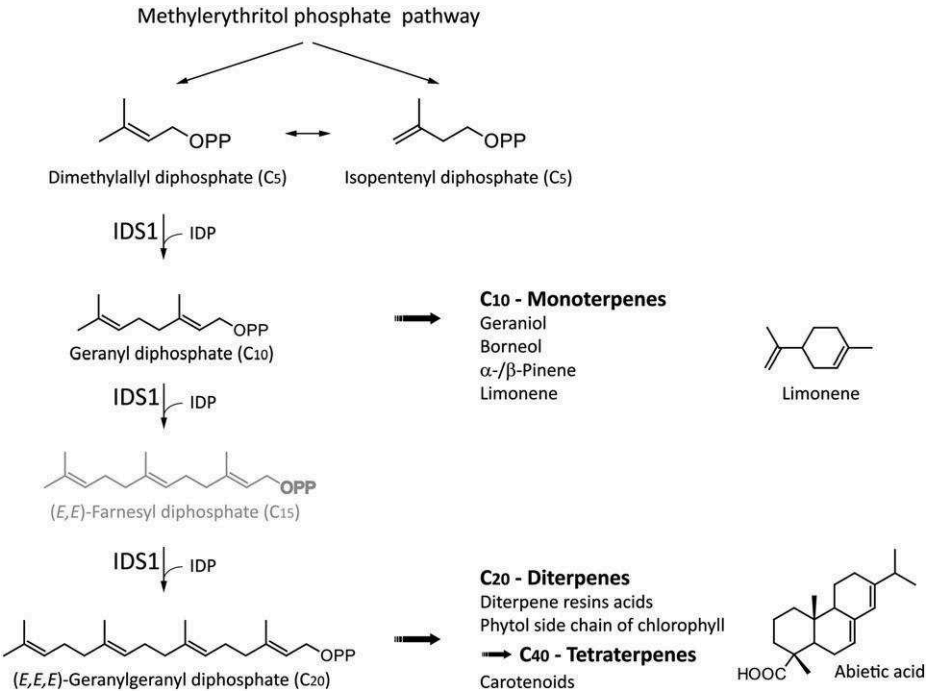


Figure 1. Outline of terpenoid biosynthesis. The schematic diagram depicts the reactions carried out by short-chain IDS1 from *P. abies*. The five-carbon building blocks IDP and DMADP, produced by the plastidial MEP pathway, are the substrates for IDS1. Sequential condensations of DMADP with one to three molecules of IDP mediated by IDS form GDP, FDP, or GGDP. The different prenyl diphosphates are then converted into representatives of the different terpene classes: GDP to mono-terpenes and GGDP to diterpenes and tetraterpenes. FDP is produced by IDS1 as an intermediate but is not released. OPP, diphosphate moiety.

Among the best known terpenes functioning in antiherbivore defense are the oleoresin components of conifers such as spruce (*Picea* spp.) that are stored in specialized resin ducts and deter insects such as bark beetles and their associated fungi (Erbilgin *et al.* 2006; Keeling and Bohlmann, 2006a, 2006b; Zhao *et al.* 2011; Schiebe *et al.* 2012). The oleoresin is composed mainly of monoterpenes (C10) and diterpene acids (C20), with very minor amounts of sesquiterpenes (Martin *et al.* 2002). This composition is congruent with the product profile of an unusual short-chain IDS of *Picea abies* (PaIDS1) that produces both GDP (C10) and GGDP (C20). When bark beetle attack is simulated by methyl jasmonate application to the bark, the expression of the IDS1 gene is increased and the translated IDS1 protein can be found in cambial cells lining the newly formed traumatic resin ducts that fill them with terpenes (Schmidt *et al.* 2010, 2011). In contrast to bark, less is known about the regulation of terpenoid biosynthesis and defenses in spruce needles. Treatment with methyl jasmonate increases the amount of stored and emitted needle terpenes (Martin *et al.* 2003), and feeding of the spruce budworm, *Choristoneura occidentalis*, induced transcript accumulation of an IDS, coding for an enzyme putatively producing GDP or GGDP (Ralph *et al.* 2006). Similar to the devastating effects of the spruce budworm in North America, the nun moth (*Lymantria monacha*) of Eurasia is also a damaging pest of spruce foliage (Wellenstein, 1942; Klimetzek and Vite, 1989; Keena *et al.* 2010). The introduction of the nun moth to North America from imported Eurasian timber poses a threat for native North American spruce, since survival and development are possible on species such as *Picea glauca* (Keena, 2003). This suggests that North American spruce should be tested for defenses against the nun moth.

Testing herbivores on conifer lines from a common genetic background in which terpene resin formation has been genetically manipulated should be a good approach to investigating the defensive role of these compounds.

In this investigation, we overexpressed IDS1 in spruce saplings to study its role in terpenoid formation and to generate altered terpene phenotypes.

2.3. RESULTS

2.3.1. Characterization of transgenic IDS1-overexpressing lines

To learn more about the potential of IDS to regulate the direction and flux through terpene biosynthesis, transgenic *P. glauca* lines were created overexpressing a *P. abies* IDS gene (PaIDS1) whose encoded protein produces GDP and GGDP. The protein sequence is more than 99% identical to that of the *P. glauca* IDS1 (PgIDS1; Supplemental Fig. S1) that was deposited in GenBank under accession number KF840686. Trans-genic 2-year-old saplings were characterized from three independent IDS1-overexpressing lines and three independent lines transformed with an empty vector. IDS1-overexpressing saplings had 500-fold higher IDS1 transcript abundance in needles compared with the empty vector control saplings, but there were no IDS1 transcript differences in bark. At the metabolite level, no significant differences were observed between IDS1-overexpressing lines and controls in monoterpenes, sesquiterpenes, diterpene resin acids, sterols, carotenoids, and chlorophylls (Supplemental Fig. S2).

2.3.2. Overexpression of IDS1 in needles increases IDS1 transcript, IDS1 protein, IDS1 enzyme activity, and isoprenyl diphosphate intermediate levels *in vivo*, but there was no change in monoterpene and diterpene resin acid contents

A single IDS1-overexpressing line (line 3) and a single empty vector control line (line 1), both 3 years old, were selected for detailed characterization to compare tissue from different growing seasons as well as different genetic backgrounds. There were no noticeable morphological differences between the two lines. The size, the number of side branches, and the coloration of the needles were all similar (Supplemental Fig. S3). After the spring flush of growth, bark and needles were harvested from both lines and divided by age. Most needles of the first year growth had already abscised. In needles, IDS1-overexpressing saplings had 300- to 700-fold higher IDS1 transcript levels than the vector control saplings, with third year needles of the overexpressing line having a 2.3-fold higher abundance of IDS1 transcript than the second year needles (Fig. 2B). The IDS1 protein was also much more abundant in needles of the IDS1-overxpressing saplings than the controls, as shown by western blots probed with specific antibodies for IDS1 (Fig. 2D). Total IDS enzyme activity as measured by in vitro assay increased substantially for GDP (C10) when comparing the needles of the IDS1-overexpressing versus empty vector control lines, about 7-fold (from 8 to 57 pmol h21 mg21 total protein) for second year needles and 17-fold (from 5 to 84 pmol h21 mg21 total protein) for third year needles. However, FDP (C15) production decreased 23-fold in IDS1-overexpressing lines (from 7 to 0.3 pmol h21 mg21 total protein) for second year needles and 10-fold (from 3 to 0.3 pmol h21 mg21 total protein) for third year needles compared with controls. On the other hand, GGDP (C20) was only produced in enzyme assays of IDS1-overexpressing saplings, with values of 10 pmol h^{21} mg^{21} total protein in second and third year needles (Fig. 2C). Despite the increased enzyme activity, individual and total amounts of mono-terpenes (0.8–1.3 mg mg^{21} fresh weight) and diterpene resin acids (1.9–2.7 mg mg^{21} fresh weight) were not significantly changed in IDS1-overexpressing saplings versus empty vector controls; sesquiterpenes were only detected in traces and, therefore, were not quantified (Fig. 2, E and F).

2.3.3. Overexpression of IDS1 increases the *in vivo* levels of prenyl diphosphate intermediates

The substantial increases in IDS1 transcript, IDS1 protein, and in vitro IDS1 enzyme activity upon over-expression in needles were inconsistent with the lack of change in terpene content. To try to reconcile this discrepancy, we also measured the amounts of GDP, FDP, and GGDP as intermediates in planta using a liquid chromatography (LC)-tandem mass spectrometry (MS/MS) method. Since large amounts of plant material were needed for this analysis, we used side branch needles not separated by age instead of stem needles. Needles of side branches have a comparable level of transcript elevation and IDS1 enzyme activity to the stem needles (Supplemental Fig. S4). In needles of IDS1-overexpressing saplings, GDP was increased from 0.07 to 0.56 nmol g^{21} fresh weight (8-fold) com-pared with vector controls, and GGDP was increased from 1.23 to 5.45 nmol g^{21} fresh weight (4.5-fold). However, FDP levels (0.07 nmol g^{21} fresh weight) were not changed (Fig. 3).

2.3.4. Overexpression of IDS1 had no major effects on bark tissue

In bark tissue, IDS1 transcripts were unchanged or had 2 to 3 times higher levels in IDS1-overexpressing saplings than controls for the first, second, and third years of growth (Fig. 4A). These increases were much less than those observed in IDS1-overexpressing needles, and there was no significant change in

total IDS1 enzyme activity when comparing IDS1-overexpressing saplings and vector controls in the bark of different years of growth (GDP as product, 40 pmol h^{21} mg^{21} total protein; FDP, 5 pmol h^{21} mg^{21} total protein; GGDP, 6 pmol h^{21} $mg^{2\ 1}$ total protein). Moreover, the levels of most prenyl diphosphates in vivo in bark were not significantly altered by IDS1 overexpression. The amounts of GDP (0.1 nmol $g^{2\ 1}$ fresh weight) and FDP (0.16 nmol g^{21} fresh weight) did not differ between IDS1-overexpressing saplings and vector controls in-dependent of the age. GGDP levels increased 1.6- and 1.9-fold with IDS1 overexpression for the first and second year growth, respectively, but these increases were much less than in the needles (Fig. 3). As for the needles, no significant differences in monoterpene (4.5–7.5 mg g^{21} fresh weight), sesquiterpene (75–185 mg g^{21} fresh weight), and diterpene resin acid (8.5–12.5 mg g^{21} fresh weight) amounts were observed between IDS1 overexpression and control lines (Fig. 4, B–F; Supplemental Fig. S5).

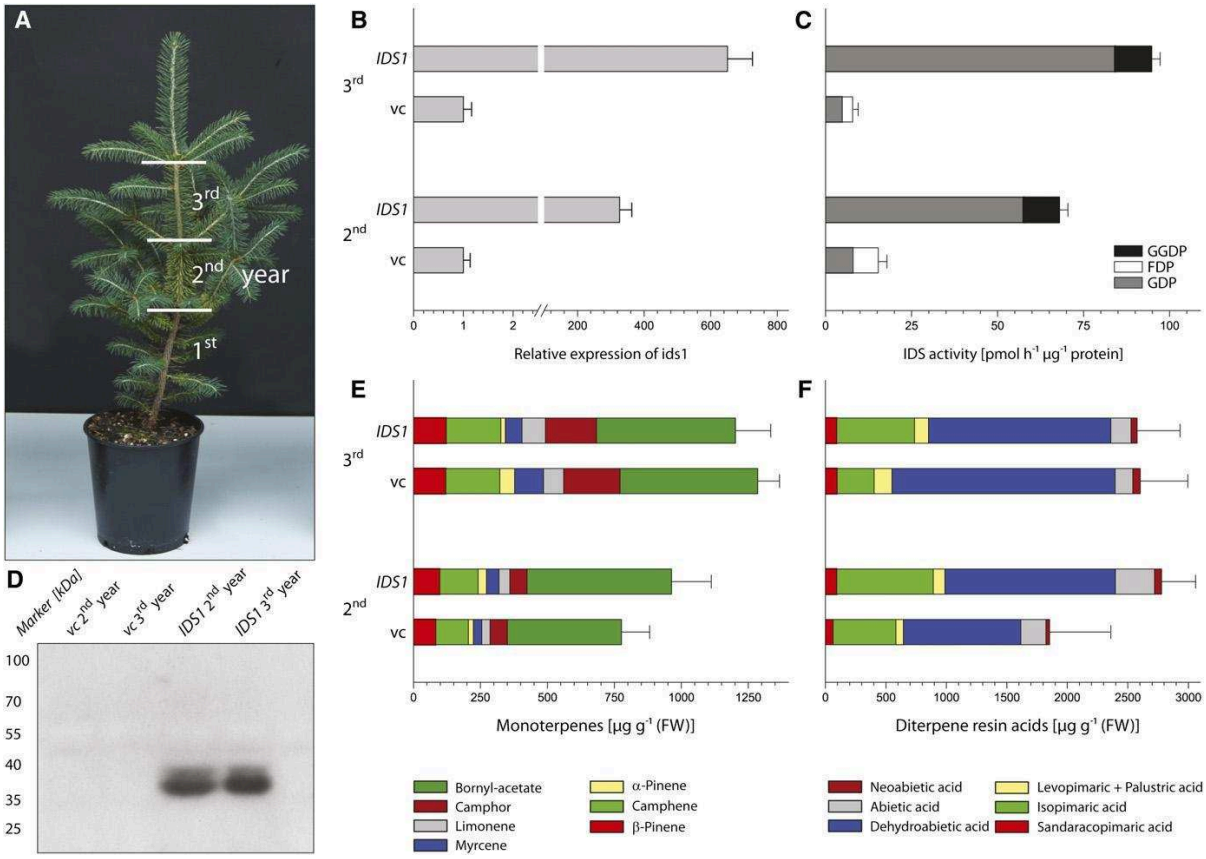


Figure 2. Detailed characterization of needles from 3-year-old *P. glauca* IDS1 in comparison with an empty vector control (vc) with respect to second- and third-year growth. A, Photograph of a sapling showing areas of first-, second-, and third-year growth on the main stem. Second- and third-year needles were sampled; first-year needles had abscised. B, Relative expression of IDS1 as determined by quantitative PCR. C, Total IDS enzyme activity measured in vitro forming GDP (C10), FDP (C15), and GGDP (C20) as determined by LC-MS/MS. D, IDS1 protein abundance measured with a specific antibody by western-blot analysis. E and F, Monoterpene (E) and diterpene resin acid (F) contents as quantified with GC-FID. Data are means 6 SD of measurements from five plants of each line. FW, Fresh weight.

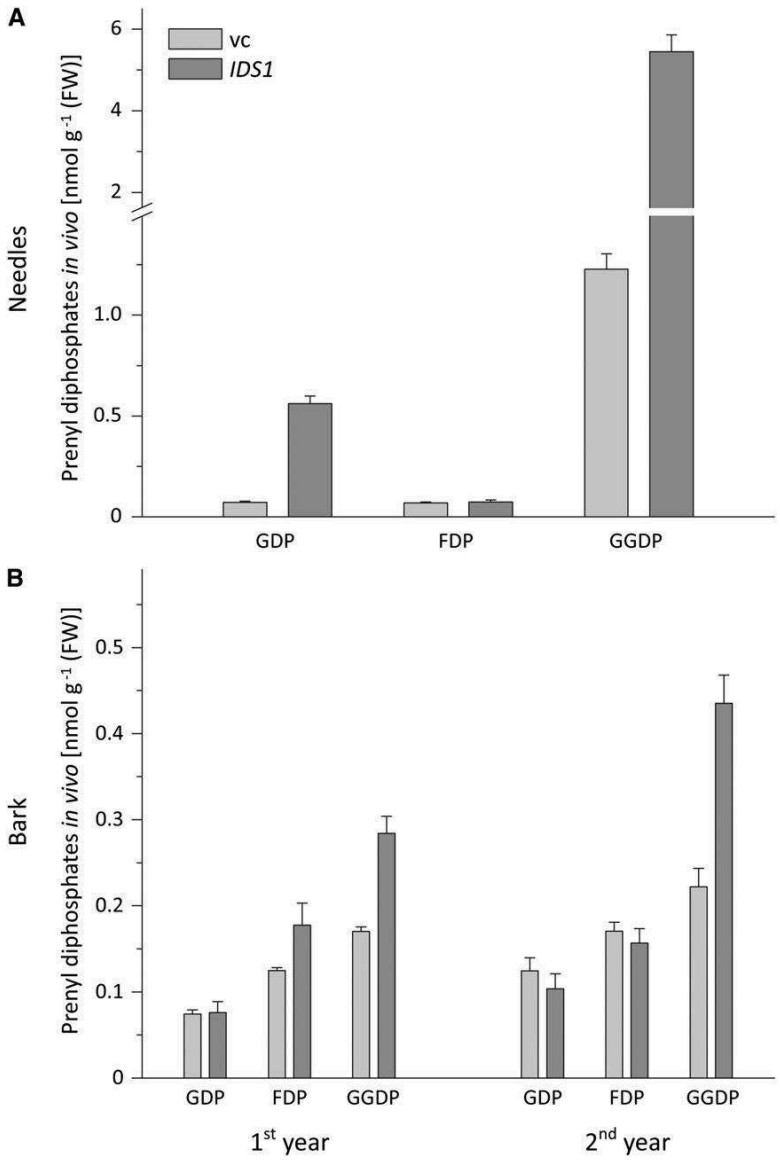


Figure 3. Quantification of in vivo levels of the isoprenoid pathway intermediates GDP, FDP, and GGDP in tissues of *P. glauca* IDS1 and an empty vector control line (vc). Measurements were per-formed on needles (A) and bark (B) by LC-MS/MS. Data are means 6 SD of measurements from five plants of each line. FW, Fresh weight

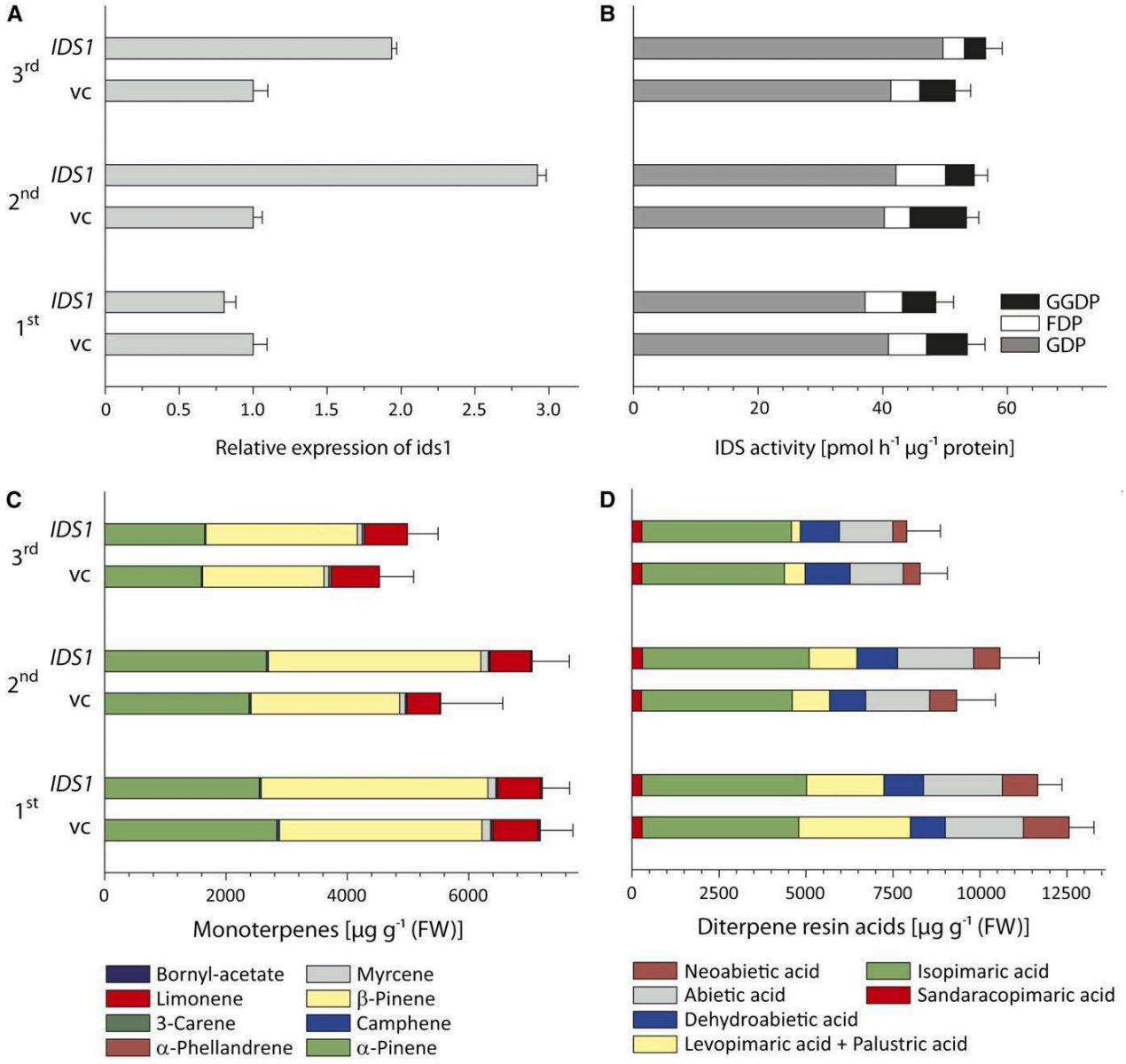


Figure 4. Detailed characterization of bark from 3-year-old *P. glauca* IDS1 in comparison with an empty vector control (vc) with respect to bark from first-, second-, and third-year growth transgenic saplings. A, Relative expression of IDS1 as determined by quantitative PCR. B, Total IDS enzyme activity measured in vitro forming GDP (C_{10}), FDP (C_{15}), and GGDP (C_{20}) as de-termined by LC-MS/MS. C and D, Monoterpene (C) and diterpene resin acid (D) contents as quantified with GC-FID. The minor monoterpenes are additionally shown separately in Supplemental Figure S5. Data are means 6 SD of measurements from five plants of each line. FW, Fresh weight

2.3.5. Overexpression of IDS1 did not affect terpenoids of primary metabolism

The effects of IDS1 overexpression were also investigated on selected terpenes of primary metabolism, such as sterols, carotenoids, and chlorophylls. For carotenoids and chlorophylls, the first and second years of growth were used, and for sterol analysis, the third year of growth was used. Relative amounts of all three metabolites did not change between vector control and IDS1-overexpressing saplings in either the bark or the needles (Fig. 5).

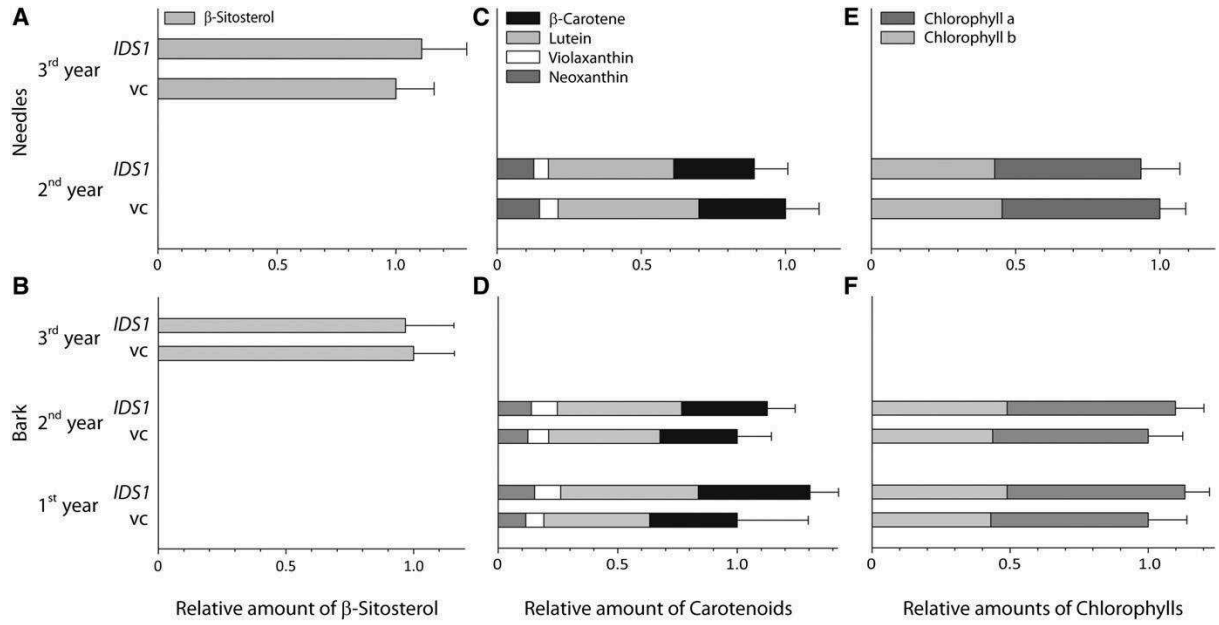


Figure 5. Quantification of other selected isoprenoids in *P. glauca* IDS1 compared with an empty vector control line (vc) with respect to first-, second-, and third-year needles and bark. A and B, Relative content of b-sitosterol, a predominant plant sterol, measured by GC-FID. The resulting peak areas, calibrated using the internal standard ergosterol, were expressed relative to the area of the vector control, set to 1. C and D, Relative content of major carotenoids was determined by HPLC with diode-array detector at 455 nm. For each organ and year, the integrated peak areas are expressed relative to that of the vector control, set to 1. E and F, Relative chlorophyll content was determined by HPLC with diode-array detector at 650 nm. For each organ and year, the integrated peak areas are expressed relative to that of the vector control, set to 1. Data are means 6 SD of measurements from five plants of each line.

2.3.6. Overexpression of IDS1 leads to the accumulation of geranylgeranyl fatty acid esters

Instead of increasing the content of monoterpenes and diterpene resin acids, IDS1 overexpression in *P. glauca* was found to dramatically increase the amounts of esters of geranylgeraniol with fatty acids in comparison with empty vector controls. Palmitic acid (C16:0), anteisoheptadecanoic acid (14-methylhexadecanoic acid [aiC17:0]), linoleic acid (C18:2), oleic acid (C18:1), and stearic acid (C18:0) were identified as the major components of the acid moieties of the esters (Fig. 6). The occurrence of these esters was initially indicated by the presence of geranylgeraniol in saponified needle extracts of

IDS1-overexpressing saplings prepared for sterol quantification. This compound was not found in saponified needle extracts of vector control saplings (Supplemental Fig. S6) or after alkaline phosphatase treatment (Supplemental Fig. S7). In addition, high amounts of fatty acids were detected in IDS1-overexpressing saplings during a diterpenoid extraction procedure that included methylation with the reagent trimethylsulfonium hydroxide (TMSH), which catalyzes transesterifications (Butte, 1983), but not after treatment with diazomethane, which methylates only free acids (Christie, 2010). Mass spectrometry (MS) and NMR measurements in comparison with those of synthesized standards of geranylgeranyl heptadecanoate and geranylgeranyl octadecanoate confirmed the presence of these and related geranylgeranyl fatty acid esters (Fig. 6B; Supplemental Fig. S8). Final confirmation of the structure of the fatty acid moiety required purification of the geranylgeranyl esters followed by transesterification with TMSH to form fatty acid methyl esters, which were compared with commercially available fatty acid methyl ester standards.

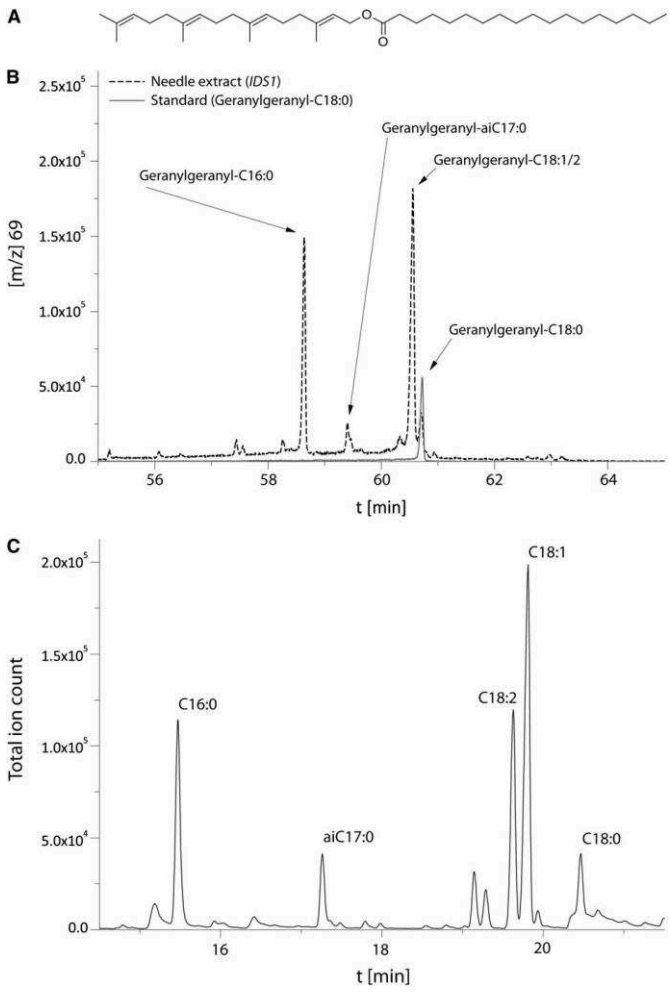


Figure 6. Evidence for the accumulation of geranylgeranyl fatty acid esters in IDS1-overexpressing *P. glauca* saplings. A, Structure of geranylgeranyl stearate (geranylgeranyl-C18:0). B, GC-MS chromatogram of a needle extract overlaid with a chromatogram of synthetic geranylgeranyl-C18:0. C, GC-MS chromatogram showing fatty acid methyl esters derived from the hydrolysis of isolated geranylgeranyl fatty acid esters. The peaks just to the left of C18:2 are impurities generated by derivatization with N-methyl-N-(trimethylsilyl)-trifluoroacetamide and not fatty acid esters. The abbreviation "aiC17:0" stands for 14-methylhexadecanoate, an anteiso fatty acid that is gymnosperm specific.

The geranylgeranyl fatty acid esters were major constituents of needles of IDS1-overexpressing saplings, with 12.2 mg g^{21} in the second year of growth, 7.76 mg g^{21} in the third year of growth, and 6.19 mg g^{21} in needles from side branches not subdivided according to their age. Their composition was dominated by esters with either C18:1 or C18:2 and C16:0 and lesser amounts of esters with aiC17:0 and C18:0 (Table I). In bark of IDS1-overexpressing saplings or in bark and needles of empty vector control saplings, these esters were not detectable in amounts greater than 0.5 mg g^{21} (Table I; Supplemental Fig. S9). To

determine if the accumulation of these prenyl esters is solely a consequence of transformation in the genetic background of *P. glauca*, IDS1-overexpressing saplings of *P. abies* were also produced. Needles of 1-year-old *P. abies* saplings had a total amount of geranylgeranyl esters of 8.35 mg g^{21}, similar to quantities present in transformed *P. glauca*.

Table I. Accumulation of geranylgeranyl (GG) fatty acid esters in transgenic lines of P. glauca and P. abies overexpressing IDS1 or empty vector controls

Genotype	Tissue	GG-C16:0	GG-aiC17:0	GG-C18:1/C18:2	GG-C18:0	Total
				mg g21 fresh wt		
P. glauca IDS1	Needles third-year, main stem	5.24±0.14	0.88±0.13	5.03±0.35	1.05±0.17	12.21±0.63
P. glauca IDS1	Needles second-year, main stem	3.05±0.42	0.78±0.06	3.28±0.41	0.65±0.08	7.76±0.89
P. glauca IDS1	Needles, side branch	3.32±0.69	0.80±0.17	3.53±0.73	0.69±0.14	8.35±1.59
P. glauca IDS1	Bark	<0.0002	<0.00002	<0.0002	<0.0001	<0.0005
P. glauca vector control	Needles, side branch	<0.0001	<0.00001	<0.0001	<0.00005	<0.00025
P. glauca vector control	Bark	<0.0002	<0.00002	<0.0002	<0.0001	<0.0005
P. abies IDS1	Needles, main stem	1.76±0.07	0.7±0.01	3.51±0.07	0.23±0.01	6.19±0.14

2.3.7. Geranylgeranyl fatty acid esters decrease the growth and survival of a spruce insect herbivore

Geranylgeranyl fatty acid esters have previously been reported at low levels in *P. abies* (Ekman, 1980). Their occurrence in large quantities in the needles of our IDS1-overexpressing line made it possible to test whether, like many other terpenes, they might have a role in plant defense. For this purpose, larvae of the nun moth, an insect that feeds on the foliage of conifers and other trees, were offered foliage from IDS1-overexpressing, empty vector control, and wild-type saplings. Larval survival and weight 21 d after hatching were significantly lower on IDS1-overexpressing plants than on either the empty vector or wild-type controls (P , 0.005, log-rank test; P , 0.02, Student's t test; Fig. 7, A and B). Developmental time, time from hatching to pupation, and time from pupation until adulthood did not vary between the treatments (Supplemental Fig. S10). Sex ratios of emerged adults were also the same, with an almost 50:50 ratio for larvae feeding on IDS1-overexpressing and control foliage (Supplemental Fig. S11).

To confirm that the higher mortality from feeding on IDS1-overexpressing plants is actually caused by the occurrence of geranylgeranyl fatty acid esters, branches from wild-type *P. glauca* saplings were treated with geranylgeranyl stearate dissolved in hexane or hexane as a control. Larvae fed on foliage amended with this purified geranylgeranyl fatty acid ester had a significantly lower survival rate than when fed on controls (P, 0.001, log-rank test; Fig. 7C).

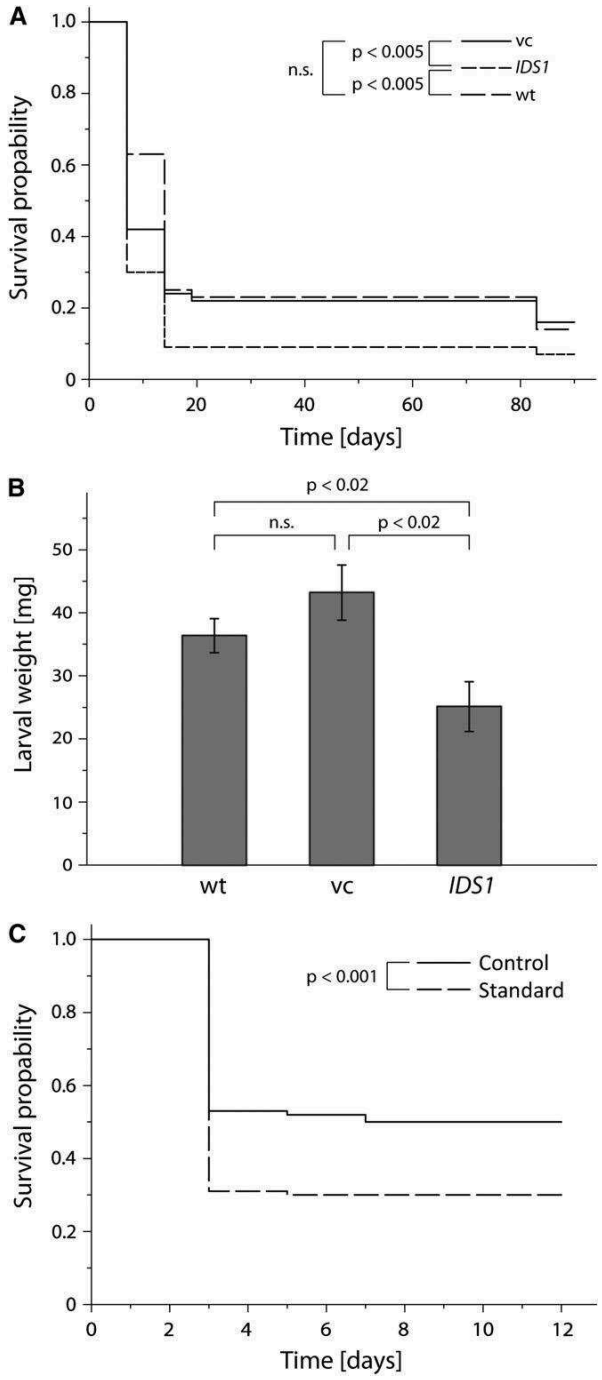


Figure 7. Effect of geranylgeranyl fatty acid esters on the performance of larvae of the nun moth. A, Kaplan-Meier plot of larval survival after feeding on foliage of IDS1, empty vector control (vc), or wild-type (wt) saplings. B, Larval mass after 24 d of feeding on the three genotypes. The IDS1-overexpressing saplings contain levels of geranylgeranyl fatty acid esters that are over 10^4 times those in either of the control lines. C, Kaplan-Meier plot of larval survival after feeding on foliage dipped in a hexane solution of synthesized geranylgeranyl stearate or dipped in a hexane control. n.s., Not significant

2.4. DISCUSSION

IDS functions at branch points of terpenoid biosynthesis, catalyzing the formation of intermediates of varying chain length, such as GDP (C_{10}), FDP (C_{15}), and GGDP (C_{20}). However, only scattered information is available about the role of IDS in controlling direction and flux through these pathways. IDS1 isolated from *P. abies* catalyzes the condensation of the C_5 units IDP and DMADP into GDP and GGDP, but not FDP (Schmidt *et al.* 2010). The C_{10} and C_{20} intermediates, GDP and GGDP, are precursors for the monoterpene hydrocarbons and diterpene resin acids, respectively, the two major classes of terpenes in conifer oleoresin. *P. abies* IDS1 has been suggested to have a major role in conifer oleoresin formation, because of the strong correlations of transcript and protein with the synthesis of resin terpenes in traumatic ducts after methyl jasmonate application (Schmidt *et al.* 2010, 2011). Here, transgenic spruce lines overexpressing IDS1 were used to test how increasing enzyme activity would affect terpene amount and composition.

2.4.1. Overexpression of IDS1 does not increase the content of typical resin monoterpenes and diterpenes

Overexpression of IDS1 under the control of the maize (*Zea mays*) ubiquitin promoter led to a massive (up to 700-fold) increase in IDS1 transcript level in needles but only a 2- to 3-fold increase in bark. Previous measurements on wild-type saplings had shown that IDS1 transcripts were about 50-fold higher in bark, the site of extensive terpene resin synthesis and storage, than in needles (Schmidt *et al.* 2010). The strength of the ubiquitin promoter might not be sufficient to increase transcription much further in bark, but in needles, where basal transcription is very low, this promoter could enhance transcription quite significantly. For successful overexpression in bark, another promoter should be chosen, or better, an as-yet-unidentified promoter specific for oleoresin production in ducts.

The elevated transcript levels in needles increased the amount of IDS1 protein, leading to a higher IDS1 enzyme activity and higher in planta levels of the products GDP and GGDP. However, FDP did not increase in planta in the needles. The reduction of FDP in the in vitro enzyme assays can be attributed to the ability of IDS1 to use FDP as a substrate for the production of GGDP (Schmidt *et al.* 2010). Despite the increase in IDS1 protein activity and GDP and GGDP, no significant changes were detected in terpene content, with the exception of a dramatic increase in geranylgeranyl fatty acid esters. The quantities of resin monoterpenes, sesquiterpenes, and diterpene resin acids did not increase in *P. glauca* and are in the same range as already reported for *P. abies* (Martin *et al.* 2002, 2003). In addition, there were no changes in the levels of the primary isoprenoids measured, including sterols, chlorophylls, and carotenoids. While overexpression of IDS1 was able to significantly increase the prenyl diphosphate intermediates GDP and GGDP, the lack of increased flux to the resin monoterpenes and diterpenes, respectively, may be due to the low activity of the next step of terpene biosynthesis, catalyzed by terpene synthases.

Another limitation to terpene accumulation in these plant lines may be the broad targeting of IDS1 overexpression under the control of the maize ubiquitin promoter. In wild-type spruce, this *P. abies* gene and genes encoding later enzymes of terpene resin biosynthesis in spruce are localized in bark cells surrounding resin ducts (Abbott *et al.* 2010; Schmidt *et al.* 2010). Here, the resin components are formed and then secreted into the ducts. Resin terpenes in spruce needles are stored in resin ducts (Weng and Jackson, 2000) and thus are likely also produced in the cells immediately surrounding these structures. Therefore, a general overexpression of IDS1 throughout the needles may not lead to an increase in terpenoids because of the lack of later pathway enzymes in most cells, the absence of a location to store

the products, or the missing ability to transport any terpenes produced toward existing storage structures. Instead, the buildup of the intermediate GGDP, but not of GDP, is diverted to the production of geranylgeranyl fatty acid esters.

Transgenic manipulation of IDS expression in other species has had a variety of results. In Arabidopsis (*Arabidopsis thaliana*), knockout of both FDP synthases led to an early developmental arrest of seeds (Closa *et al.* 2010), while overexpression of Arabidopsis FDPS1 induced oxidative stress, cell death, and lesions due to altered cytokinin levels (Masferrer *et al.* 2002; Manzano *et al.* 2004, 2006). In *Artemisia annua*, FDP synthase overexpression increased the amount of the antimalarial sesquiterpene artemisinin but also had effects on plant morphology (Han *et al.* 2006; Banyai *et al.* 2010). In contrast to the cytosol-localized FDP synthases, manipulation of the plastid-localized GDP and GGDP synthases appears to alter terpene levels with fewer other effects on the plant, indicating differences between cytosolic and plastidal regulation of terpene biosynthesis. For example, overexpression of GDP synthase in *Mentha x piperita* increased the amount of monoterpenes (Lange *et al.* 2011), and overexpression of GGDP synthase in *Salvia miltiorrhiza* hairy root cultures increased the amount of the diterpene tanshinone (Kai *et al.* 2011), while the loss of GGDP synthase activity in *Nicotiana attenuata* decreased the level of defensive diterpene glycosides (Jassbi *et al.* 2008). In fact, when an FDP synthase, normally found in the cytosol, was expressed in the plastid (together with a terpene synthase), higher terpene amounts were obtained than when the same enzymes were expressed in the cytosol (Wu *et al.* 2006). Overexpression of a GDP synthase in both compartments had nearly no influence on terpene amount (Wu *et al.* 2006), because this particular GDP synthase was, in fact, subsequently shown to produce medium/ long-chain prenyl diphosphates instead of GDP (Hsieh *et al.* 2011). In this work, overexpression of the IDS1 gene, which has a plastid-targeting signal peptide (Schmidt *et al.* 2010), had no impact on the content of resin mono-terpene and diterpenes, but flux was diverted to prenyl esters. Thus, constitutive overexpression of an IDS does not seem to be an appropriate way to increase the amount of resin terpenes. An RNA interference approach is currently under way to further investigate the role of IDS in terpene biosynthesis.

To the best of our knowledge, this work provides the first in planta quantification of GDP, FDP, and GGDP together. GDP has been previously quantified in two publications on spruce and other woody plants, giving concentrations 200-fold higher than what we obtained (Nogués *et al.* 2006; Ghirardo *et al.* 2010). These previous values were obtained by acid hydrolysis of the diphosphate moiety to form geraniol, which rearranges to linalool, quantified by proton-transfer reaction MS. The authors themselves (Ghirardo *et al.* 2010) recognized that their values might be overestimates of in planta GDP levels, since linalool might be formed from other substances under acidic conditions. For GGDP, 10-fold higher amounts in etiolated oat (*Avena sativa*) seedlings were reported (Benz *et al.* 1983). Large amounts of GGDP might be present in etiolated seed-lings to form chlorophyll and initiate photosynthesis rapidly on illumination. The concentration of DMADP reported (Nogués *et al.* 2006; Ghirardo *et al.* 2010) is over 100- to 1,000-fold greater than our measurements of GDP, FDP, and GGDP. If this disparity is correct, it indicates a metabolic bottleneck in terpenoid metabolism after the MEP or MVA pathway and prior to the action of IDS enzymes. In some isoprene-emitting woody plant species, including spruce, a large pool of DMADP may be critical in supplying substrate for isoprene bio-synthesis (Monson *et al.* 2013).

To make terpenes larger than C10, more IDP than DMADP is needed, and it is assumed that the ratio be-tween IDP and DMADP is regulated by isopentenyl diphosphate isomerase (Berthelot *et al.* 2012). In kudzu (*Pueraria lobata*) leaves, which make high levels of isoprene (C_5), this ratio was determined to be about 0.5 (Zhou *et al.* 2013), but it might be higher in plants that make high levels of terpenes with 10 or more carbon atoms. The greater amount of GGDP in our measurements, especially in needles, in comparison with that of GDP and FDP may be a result of GGDP's role as a precursor for GAs, carotenoids, and the phytol side chain of chlorophyll in photosynthetically active tis-sue. No detailed data are yet

available about the regulatory interactions between the IDS enzymes and isopentenyl diphosphate isomerase and isoprene synthase. However, it was recently demonstrated that IDP and DMADP have inhibitory effects on the initial step of the MEP pathway, 1-deoxy-D-xylulose-5-phosphate synthase (Banerjee *et al.* 2013). Thus, future efforts should be made to determine whether GDP, FDP, or GGDP are also inhibitory to this enzyme or other MEP or MVA pathway enzymes as well as to determine how metabolic flux is controlled among the IDS-catalyzed steps of terpene biosynthesis.

2.4.2. Elevated levels of GGDP are diverted to form geranylgeranyl fatty acid esters

IDS1-overexpressing spruce saplings showed no significant changes in their content of typical *P. glauca* resin monoterpenes and diterpene acids; instead, they exhibited a dramatic increase in a diversion product, esters of geranylgeraniol with various fatty acids. Given a typical concentration of 2.5 to 3.0 mg g^{21} fresh weight diterpene acids in spruce needles, the amount of GGDP diverted to the esters in IDS1-overexpressing plants is about twice the amount of GGDP employed for diterpene acid formation (assuming about half of the mass of geranylgeranyl fatty acid esters, 4–6 mg g^{21} fresh weight, results from geranylgeraniol). Thus, there has been at least a 3-fold increase in GGDP production in the transgenic plants, which is in broad agreement with the 4.5-fold increase in GGDP measured *in vivo*.

Despite elevated levels of isoprenoid pathway inter-mediates, such as GGDP, we did not detect any increase in other primary terpenoids, such as phytol and carotenoids, also formed from GGDP (Fig. 8), indicating that formation of these products is tightly regulated (Cazzonelli and Pogson, 2010). Although we did not measure the levels of the GAs, another group of GGDP metabolites, an increase in these hormones also seems unlikely. First, no obvious morphological differences were found between the transgenic saplings and the controls (Supplemental Fig. S3). Second, GA biosynthesis is known to be regulated mainly by transcriptional changes of transcripts of the early pathway enzymes, entcopalyl diphosphate synthase and entkaurene synthase, rather than the level of GGDP (Silverstone *et al.* 1997; Hedden and Thomas, 2012).

The pathway for the formation of geranylgeranyl fatty acid esters likely proceeds from GGDP to geranylgeraniol via phosphatase cleavage (Fig. 8). A prenyl diphosphate phosphatase from Croton stellatopilosus was recently identified (but not biochemically characterized) that is part of a family of related phosphatases from eukaryotic as well as prokaryotic sources (Nualkaew *et al.* 2013). The resulting free geranylgeraniol is then esterified. In Arabidopsis, two enzymes were de-scribed as being responsible for the synthesis of esters using the more saturated C_{20} isoprenoid alcohol, phytol, instead of geranylgeraniol (Fig. 8). These are named PHYTYL ESTER SYNTHASE1 and PHYTYL ESTER SYNTHASE2 and are members of the esterase/ lipase/thioesterase family of acyltransferases (Lippold *et al.* 2012). Since both are localized to the plastids, this suggests that the entire sequence for the formation of geranylgeranyl esters via IDS1, phosphatase, and then the ester synthase is localized in plastids. For phytyl fatty acid esters, localization in the plastoglobules was shown (Gaude *et al.* 2007). An identical localization of geranylgeranyl fatty acid esters can thus be assumed, because of the close structural similarities between these compounds.

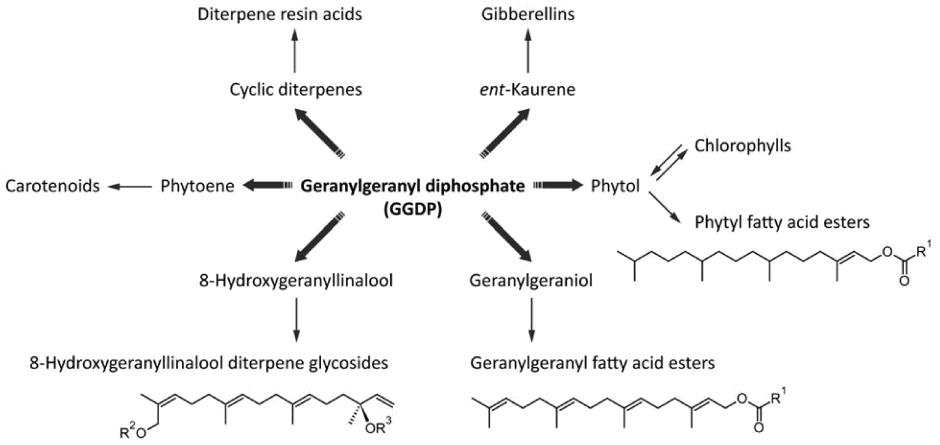


Figure 8. Metabolism of GGDP to primary metabolites, including GAs, chlorophylls, and carotenoids, and to secondary metabolites, including cyclic diterpenes, acyclic fatty acid esters, and acyclic glycosides. For phytyl and geranylgeranyl fatty acid esters, R^1 can be various fatty acids from C_{12} to C_{18} with various degrees of saturation (Ischebeck *et al.* 2006). For 8-hydroxygeranyllinalool diterpene glycosides, R^2 and R^3 are different monosaccharides or disaccharides with and without malonylation (Heiling *et al.* 2010).

2.4.3. Geranylgeranyl fatty acid esters are common in plants and function in defense

Esters of geranylgeraniol with fatty acids were already described in *P. abies* wood (Ekman, 1980), in mosses (Liljenberg and Karunen, 1978; Karunen *et al.* 1980), and in other plants (Lütke-Brinkhaus *et al.* 1985; McKibben *et al.* 1985; Jondiko and Pattenden, 1989; Reiter and Lorbeer, 2001; Biedermann *et al.* 2008). Esters with geraniol instead of geranylgeraniol are also known in rose (*Rosa* spp.) petals (Dunphy, 2006). However, no esters with geraniol were found in our transgenic *P. glauca*, although IDS1 is known to produce GDP and GGDP in vitro (Schmidt *et al.* 2010) and both intermediates were elevated in planta also, with the GGDP concentration being 12 times higher than that of GDP (Fig. 5A). The occurrence of phytyl esters is also common in plants, as mentioned above (Csupor, 1970; Liljenberg, 1977; Anderson *et al.* 1984; Pereira *et al.* 2002; Gaude *et al.* 2007), indicating that esterification of prenyl alcohols to fatty acid esters is a widespread phenomenon. The unusual anteiso fatty acid found as a component of these esters, anteiso-heptadecanoic acid (14-methylhexadecanoic acid), has also already been reported from other conifers of the *Pinaceae* (Wolff *et al.* 1997, 2001).

In contrast to phytyl esters, which are known to be involved in chlorophyll a and b synthesis and in senescence (Ischebeck *et al.* 2006), the role of geranylgeranyl fatty acid esters had not yet been investigated. Here, we found these compounds to reduce the growth and survival of the conifer needle-feeding larvae of the nun moth, suggesting that they function in plant defense against herbivores (Fig. 7). Their toxicity may be a consequence of hydrolysis in the insect. Gut extracts of the closely related species *Lymantria dispar* possess high esterase activity (Kapin and Ahmad, 1980) that could cause cleavage of the ester bond and the release of geranylgeraniol, which has been described to exert toxic effects on membranes in vitro and against *Staphylococcus aureus* (Funari *et al.* 2005; Inoue *et al.* 2005). Interestingly, another group of plant defense compounds, the hydroxygeranyllinalool glycosides found in *N. attenuata* (Jassbi *et al.* 2008; Heiling *et al.* 2010; Đinh *et al.* 2013), share a similar terpene moiety and also can be readily cleaved to release an acyclic diterpene alcohol. The toxicity of geranyllinalool by itself has been reported for ants and termites (Lemaire *et al.* 1990). In both cases, conjugation of the diterpene moiety with a sugar or fatty acid could function as a strategy for storing these toxins to prevent damage to

membranes or other plant components (Fig. 8). In fact, the overaccumulation of geranylgeraniol moieties as fatty acid esters in IDS1-overexpressing plants may be essential to avoid auto-toxicity. Further experiments are planned to investigate the occurrence of geranylgeranyl fatty acid esters in spruce and their role in defense against other herbivores and pathogens.

2.5. Materials and Methods

2.5.1. Chemicals

Geranylgeraniol, octadecanoyl chloride, pentadecanoyl chloride, 4-(dimethylamino) pyridine, para-cymene, iodine, alkaline bovine phosphatase, Supelco 37 Component FAME Mix, chloroform, dichloromethane, isopentenyl diphosphate, dimethylallyl diphosphate, ammonium bicarbonate, tert-butyl methyl ether (TBME), and acetonitrile (LC-MS grade) were purchased from Sigma-Aldrich. N-Methyl-N-(trimethylsilyl) trifluoroacetamide and TMSH were ordered from Macherey-Nagel. Methyl 14-methylhexadecanoate was purchased from Larodan Fine Chemicals, and tetrahydroabietic acid was from Wako Pure Chemical Industries.

2.5.2. Plant material

Agrobacterium tumefaciens-mediated transformation of a *Picea glauca* embryogenic cell culture overexpressing IDS1 from *Picea abies* (Schmidt *et al.* 2010) was carried out as described previously by Klimaszewska *et al.* (2001) and Hammerbacher *et al.* (2011), with the gene under the control of a maize (*Zea mays*) ubiquitin promoter. Somatic embryogenesis and plant regeneration of transgenic saplings were carried out, and plants were transferred to soil. For the analysis of geranylgeranyl fatty acid ester accumulation in *P. abies*, an identical transformation protocol was used. In the case of *P. glauca*, three independent transformed lines plus three empty vector control lines were characterized initially, using four plants per line. For later in-depth characterization, one line each from transformants and controls were chosen using five plants per line.

2.5.3. Monoterpene, sesquiterpene, and diterpene analysis

The protocol was adapted from Lewinsohn *et al.* (1993). In brief, 100 mg of frozen plant material was ground in 1 mL of TBME with 57 mg mL^{-1} p-cymene and 46 mg mL^{-1} tetrahydroabietic acid as internal standards and extracted under continuous shaking for 24 h. The extract was removed, washed with 0.3 mL of 0.1 M $(NH_4)_2CO_3$, pH 8.0, and dried by using a Pasteur pipette filled with 100 mg of Na_2SO_4. The Na_2SO_4 column was further washed with 1 mL of TBME. To 0.4 mL of extract, 50 mL of TMSH was added for methylation of diterpenoid resin acids, which were subsequently analyzed by gas chromatography (GC)-MS and GC-flame ionization detection (FID). The rest was used for monoterpene and sesquiterpene analysis. GC conditions were as described by Schmidt *et al.* (2011). All terpenes were quantified on a fresh weight basis; the ratio of fresh weight to dry weight is about 3.5 for needles and 4.0 for bark tissues.

2.5.4. Isolation, purification, and MS analysis of geranylgeranyl fatty acid esters

Plant material was extracted with hexane:diethyl ether (9:1, v/v) in a ratio of 100 mg of tissue to 4 mL of solvent for 24 h under continuous shaking. The solvent was evaporated to dryness under a stream of nitrogen. The residual material was taken up in 0.5 mL of hexane:diethyl ether (9:1, v/v), loaded on a 3-mL, 500-mg SPE cartridge (CHROMABOND SiOH; Macherey-Nagel), and equilibrated with 3 mL of hexane:diethyl ether (9:1, v/v). Further elution was done with the same solvent. The first 2 mL was collected, evaporated to dryness, and taken up in 200 mL of chloroform. An Agilent 6890 series GC instrument with an Agilent 5973 mass spectrometer and a Zebron ZB-5HT column (30 m 3 0.25 m 3 0.25 mm; Phenomenex) were used for detection. One microliter of sample was injected in split mode (1:10) with an injector temperature of 310°C and a flow rate of 1 mL min^{21} helium. Initial oven temperature was 50°C, held for 4 min, then raised by 5°C min^{21} to 330°C, and held there for 15 min. ChemStation G1701 was used for data analysis. For quantification, geranylgeranyl heptadecanoate was used as an internal standard at a concentration of 25 mg mL^{-1}. Quantification was done using the mass-to-charge ratio (m/z) 69 fragment ion and assuming a relative response rate of 1. GC-MS electrospray ionization spectra can be found in Supplemental Figure S12.

2.5.5. NMR analysis of geranylgeranyl fatty acid esters

All NMR spectra were measured on a Bruker Avance 500 NMR spec-trometer (Bruker Biospin), operating at 500.13 MHz for ^{1}H and 125.75 MHz for ^{13}C. A triple resonance inverse cryoprobe (5 mm) was used to measure spectra at a probe temperature of 300 K. Spectra are referenced to tetramethylsilane at d 0 ppm. For data processing and spectrometer control, TOPSPIN version 2.1 was used. The all-trans-configuration of the geranylgeranyl moiety could be deduced from chemical shifts in accordance with previously published data and from selective rotating frame nuclear Overhauser effect correlation spectroscopy experiments (Tanaka *et al.* 1982; Tanaka and Hirasawa, 1989).

2.5.6. Synthesis of geranylgeranyl stearate as a standard in geranylgeranyl fatty acid ester analysis

The synthesis was modified after a published method (Vassão *et al.* 2007). Quantities of 100 mg of geranylgeraniol (purity, 85%) and 30 mg of 4-(dimethylamino)-pyridine were dissolved in 25 mL of dichloromethane on an ice bath, and 100 mg of either octadecanoyl or heptadecanoyl chloride was added. The solution was stirred further for 24 h at room temperature. To stop the reaction, 20 mL of a saturated NaCl solution were added. The organic phase was dried with Na2SO4 and concentrated using a rotary evaporator. The reaction products were dissolved in 1 mL of hexane:diethyl ether (9:1, v/v) and purified using a SPE cartridge (CHROMABOND SiOH; Macherey-Nagel) equilibrated and eluted with hexane:diethyl ether (9:1, v/v). The eluate was fractionated and tested for purity by applying 10 mL of each fraction to a silica gel thin-layer chromatography plate, which was developed in hexane:diethyl ether (9:1, v/v) and stained with iodine vapor.

2.5.7. Sterol analysis

One hundred milligrams of plant material was extracted with 4 mL of dichloromethane:methanol (2:1, v/v), including 60 mg mL^{-1} ergosterol as an internal standard, for 2 h at room temperature under continuous shaking. The solvent was evaporated under a stream of nitrogen, and the sample was dissolved in 2 mL of 2 M KOH in ethanol:water (3:1, v/v) followed by an incubation for 2 h at 60°C. After 2 mL of water was added, extraction was done three times with 2 mL of diethyl ether. The organic phases were combined and evaporated to dryness, and 100 mL of tetrahydrofuran and 100 mL of N-methyl-N-(trimethylsilyl) trifluoroacetamide were added. An Agilent 6890 series GC device with an Agilent 5973 mass spectrometer and a Zebron ZB-5MSi column (30 m 3 0.25 m 3 0.25 mm; Phenomenex) was used for

analysis. One microliter of sample was injected in split mode 1:20 with an injector temperature of 280°C and a flow rate of 1 mL min^{-1} helium. Initial oven temperature was 200°C, held for 2 min, then raised by 15°C min21 to 280°C and by 2°C min^{-1} to 300°C, and held there for 4 min. ChemStation G1701 was used for data analysis and integration of the peak areas.

2.5.8. Carotenoid and chlorophyll analysiS

Extraction of 100 mg of plant material was carried out with 2 mL of acetone in brown glass vials at 4°C for 24 h under continuous shaking. An 800-mL portion of the extract was diluted with 200 mL of water, and 50 mL was injected on an Agilent 1100 HPLC instrument using a Supelcosil LC-18 column (7.5 cm 3 4.6 mm 3 3 mm; Sigma-Aldrich). Column temperature was kept at 20°C with a flow rate of 1.5 mL min^{-1}. Buffer A was 1 mM NaHCO3 and buffer B was acetone. Starting conditions were 65% B, held for 4 min, followed by an increase to 90% B in 8 min and to 100% B in 8 min, held for 2 min. Carotenoids were detected at 455 nm and chlorophyll at 650 nm. For data analysis and integration of peak areas, ChemStation for LC 3D was used.

2.5.9. SDS-PAGE and western blot

Plant material was extracted as described (Nagel *et al.* 2012), and 34 mg of total protein was separated on a 12% SDS-PAGE gel under reducing conditions and transferred to an Immobilon-P polyvinylidene difluoride membrane (Merck Millipore). The membrane was blocked with 5% skim milk powder in PBS-T (phosphate-buffered saline, 137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 2.0 mM KH2PO4, pH 7.4, and 0.05% [v/v] Tween 20). Thereafter, the membrane was incubated with an IDS1 specific polyclonal antibody serum (Schmidt *et al.* 2010) diluted 1:500 in blocking solution. The secondary anti-body was an anti-rabbit horseradish peroxidase (Sigma-Aldrich) diluted 1:5,000 in PBS-T without skim milk powder. Washes between the incubation steps were done with PBS-T. Signal detection was achieved by using West Pico Chemiluminescent Substrate (Thermo Scientific) and Amersham Hyperfilm ECL (GE Healthcare).

2.5.10. Protein extraction and quantification of IDS enzyme activity

Protein extraction, enzyme assays, and analysis were carried out as de-scribed by Nagel *et al.* (2012). Data analysis was performed using Analyst Software 1.6 Build 3773 (AB Sciex Instruments).

2.5.11. Quantification of short-chain prenyl diphosphates *in planta*

A 0.75-g portion of plant material was extracted three times with 5 mL of methanol:water (7:3, v/v), including a total of 0.3 mg of geranyl S-thiolodiphosphate and farnesyl S-thiolodiphosphate each (Echelon Biosciences). Extracts were combined, and 5 mL of water was added. Extracts were purified using 150 mg (6 mL) of CHROMABOND HR-XA columns (Macherey-Nagel), conditioned with 5 mL of methanol and 5 mL of water. After application of ex-tract, the column was washed with 4 mL of water followed by 5 mL of methanol. Prenyl diphosphates were eluted with 3 mL of 1 M ammonium formate in methanol, evaporated under a stream of nitrogen to dryness, and dissolved in 250 mL of water:methanol (1:1). Quantification was done using an Agilent 1260 HPLC system (Agilent Technologies) coupled to an API 5000 triple-quadrupole mass spectrometer (AB Sciex Instruments). For separation, a ZORBAX Extended C-18 column (1.8 mm, 50 mm 3 4.6 mm; Agilent Technologies) was used. The mobile phase consisted of 5 mM ammonium bicarbonate in water as solvent A and acetonitrile as solvent B, with the flow rate set at 1.2 mL min21, and the column temperature was kept at 20°C. Separation was achieved by using a gradient starting at 5% B, increasing to 7% B in 5 min and 100% B in 1 min (0.5-min hold), followed by a change to 0% B in 1.5 min (1-min hold) before the next injection. The injection volume for samples and

standards was 2 mL; autosampler temperature was 4°C. The mass spectrometer was used in the negative electrospray ionization mode. Optimal settings were determined using standards. Levels of ion source gases 1 and 2 were set at 60 and 70 pounds per square inch (p.s.i., respectively, with a temperature of 700°C. Curtain gas was set at 30 p.s.i., and collision gas was set at 7 p.s.i., with all gases being nitrogen. Ion spray voltage was maintained at 24,200 V. Multiple reaction monitoring was used to monitor analyte parent ion-to-product ion formation: m/z 312.9/79 for GDP, m/z 380.9/79 for FDP, m/z 449/79 for GGDP, m/z 329/79 and 329/159 for geranyl S-thiolodiphosphate, and m/z 379/79 and 379/159 for farnesyl S-thiolodiphosphate. Data analysis was performed using Analyst Software 1.6 Build 3773 (AB Sciex Instruments).

2.5.12. Quantitative real-time PCR and quantitative genomic PCR

RNA isolation, complementary DNA synthesis, and quantitative PCR from needle tissue were done as described by Schmidt *et al.* (2011). Quantitative genomic PCR was done from needle tissue as described by Schmidt *et al.* (2010).

2.5.13. Cloning of PgIDS1 and alignment with PaIDS1

RNA was isolated from bark tissue of *P. glauca*, and the PgIDS1 sequence was amplified with the primers and conditions described by Schmidt *et al.* (2010). The resulting fragment was cloned into pCR 4-TOPO (Invitrogen) and transformed into Escherichia coli strain TOP10F (Invitrogen) according to the manufacturer's instructions. Positive clones were selected, and sequence analysis was carried out using an ABI 3100 automatic sequencer (Applied Biosystems). The DNAStar Lasergene program version 9.0 (MegAlign) was used to align PgIDS1 (GenBank accession no. KF840686) with PaIDS1 (GenBank accession no. GQ369788).

2.5.14. Insect assays

Eggs of nun moth (*Lymantria monacha*) were collected in fall 2011 near Herzberg/Elster, Germany, and kept at 28°C for 2 months plus 2 weeks at +4°C and then at room temperature until hatching. Hatched larvae were selected randomly for the different treatments and placed in groups of 50 larvae in Steri Vent Containers (Duchefa). Cut branches of wild-type, vector control IDS1-overexpressing spruce saplings were inserted in a water-filled 2-mL tube through a hole in the lid of each container and changed every third day. Larvae were counted once per week, and numbers were equalized every second week in each treatment group. Larval weight was determined separately for each individual after 21 d. To determine the time needed for pupation and the sex of the emerging adult, containers were checked for pupae every day after the first pupation event occurred, and pupae were placed in separate containers. In total, 300 larvae were fed on vector control and IDS1-overexpressing saplings, and 150 larvae were fed on wild-type plants.

For the feeding assay with geranylgeranyl stearate standard applied to branches from wild-type spruce saplings, ends of cut branches were placed in water-filled 2-mL tubes through a hole in the lid and swirled in hexane including 2 mg mL21 geranylgeranyl stearate or pure hexane for 10 s. Newly treated branches were placed in a container with nun moths every day, and larvae were counted on days 3, 5, 7, 9, and 12. One hundred larvae were used for each treatment.

2.5.15. Statistical analysis

The statistical significance of nun moth weight gain was tested by pairwise comparison using Student's t test. Survival curves were compared using a log-rank test.

2.5.16. Data accessibility

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession number KF840686 (PgIDS1).

2.6. ACKNOWLEDGEMENTS

We thank Dr. Paulina Dabrowska (Max Planck Institute of Chemical Ecology) for the synthesis of diazomethane, Annett Engelmann and Dr. Katrin Möller (Landeskompetenzzentrum Forst Eberswalde) for collecting and pro-viding nun moth eggs, and Ines Lassowskat (Institute of Plant Biochemistry) and Dr. Michael Reichelt (Max Planck Institute for Chemical Ecology) for useful discussions regarding prenyl diphosphate purification.

2.7. REFERENCES

Abbott E, Hall D, Hamberger B, Bohlmann J (2010) Laser microdissection of conifer stem tissues: isolation and analysis of high quality RNA, terpene synthase enzyme activity and terpenoid metabolites from resin ducts and cambial zone tissue of white spruce (*Picea glauca*). *BMC Plant Biology,* 10: 106–121.

Akhtar TA, Matsuba Y, Schauvinhold I, Yu G, Lees HA, Klein SE, Pichersky E (2013) The tomato cis-prenyltransferase gene family. *Plant Journal,* 73: 640–652.

Anderson WH, Gellerman JL, Schlenk H (1984) Effect of drought on phytyl wax esters in *Phaseolus* leaves. *Phytochemistry,* 23: 2695–2696.

Arigoni D, Sagner S, Latzel C, Eisenreich W, Bacher A, Zenk MH (1997) Terpenoid biosynthesis from 1-deoxy-D-xylulose in higher plants by intramolecular skeletal rearrangement. *Proceedings of the National Academy of Sciences of the United States of America,* 94: 10600–10605.

Banerjee A, Wu Y, Banerjee R, Li Y, Yan H, Sharkey TD (2013) Feedback inhibition of deoxy-D-xylulose-5-phosphate synthase regulates the methylerythritol 4-phosphate pathway. *Journal of Biological Chemistry,* 288: 16926–16936

Banyai W, Kirdmanee C, Mii M, Supaibulwatana K (2010) Overexpression of farnesyl pyrophosphate synthase (FPS) gene affected artemisinin content and growth of *Artemisia annua* L*. Plant Cell, Tissue and Organ Culture,* 103: 255–265.

Benz J, Fischer I, Rüdiger W (1983) Determination of phytyl diphosphate and geranylgeranyl diphosphate in etiolated oat seedlings. *Phytochemistry,* 22: 2801–2804.

Berthelot K, Estevez Y, Deffieux A, Peruch F (2012) Isopentenyl diphosphate isomerase: a checkpoint to isoprenoid biosynthesis. *Biochimie,* 94: 1621–1634.

Biedermann M, Haase-Aschoff P, Grob K (2008) Wax ester fraction of edible oils: analysis by online LC-GC-MS and GC x GC-FID. *European Journal of Lipid Science and Technology,* 110: 1084–1094.

Bohlmann J, Keeling CI (2008) Terpenoid biomaterials. Plant J 54: 656–669

Buchanan BB, Gruissem W, Jones RL (2000) Biochemistry and Molecular Biology of Plants. John Wiley and Sons, New York.

Butte W (1983) Rapid method for the determination of fatty acid profiles from fats and oils using trimethylsulphonium hydroxide for trans-esterification. *Journal of Chomatography A,* 261: 142–145.

Cazzonelli CI, Pogson BJ (2010) Source to sink: regulation of carotenoid biosynthesis in plants. *Trends in Plant Science,* 15: 266–274.

Chen DH, Ye HC, Li GF (2000) Expression of a chimeric farnesyl diphosphate synthase gene in Artemisia annua L. transgenic plants via *Agrobacterium tumefaciens*-mediated transformation. *Plant Science,* 155: 179–185.

Chen F, Tholl D, Bohlmann J, Pichersky E (2011) The family of terpene synthases in plants: a mid-size family of genes for specialized metabolism that is highly diversified throughout the kingdom. *Plant Journal,* 66: 212–229.

Christie WW (2010) Lipid Analysis: Isolation, Separation, Identification and Structural Analysis of Lipids, Ed 4. Oily Press, Bridgwater, UK.

Closa M, Vranová E, Bortolotti C, Bigler L, Arró M, Ferrer A, Gruissem W (2010) The *Arabidopsis thaliana* FPP synthase isozymes have overlapping and specific functions in isoprenoid biosynthesis, and complete loss of FPP synthase activity causes early developmental arrest. *Plant Journal,* 63: 512–525.

Csupor L (1970) [Phytol in yellowed leaves]. *Planta Medica* 19: 37–41.

Đinh ST, Gális I, Baldwin IT (2013) UVB radiation and 17-hydroxyger-anyllinalool diterpene glycosides provide durable resistance against mirid (*Tupiocoris notatus*) attack in field-grown *Nicotiana attenuata* plants. *Plant, Cell and Environment*, 36: 590–606.

Dunphy PJ (2006) Location and biosynthesis of monoterpenyl fatty acyl esters in rose petals. *Phytochemistry*, 67: 1110–1119.

Ekman R (1980) Geranylgeranyl esters in Norway spruce wood. *Phytochemistry,* 19: 321–322.

Erbilgin N, Krokene P, Christiansen E, Zeneli G, Gershenzon J (2006) Exogenous application of methyl jasmonate elicits defenses in Norway spruce (*Picea abies*) and reduces host colonization by the bark beetle *Ips typographus*. *Oecologia,* 148: 426–436.

Funari SS, Prades J, Escribá PV, Barceló F (2005) Farnesol and geranylgeraniol modulate the structural properties of phosphatidyletha-nolamine model membranes. *Molecular Membrane Biology,* 22: 303–311.

Gaude N, Bréhélin C, Tischendorf G, Kessler F, Dörmann P (2007) Nitrogen deficiency in *Arabidopsis* affects galactolipid composition and gene expression and results in accumulation of fatty acid phytyl esters. *Plant Journal,* 49: 729–739.

Gershenzon J, Dudareva N (2007) The function of terpene natural products in the natural world. *Nature Chemical Biology,* 3: 408–414.

Ghirardo A, Koch K, Taipale R, Zimmer INA, Schnitzler JP, Rinne J (2010) Determination of de novo and pool emissions of terpenes from four common boreal/alpine trees by 13CO2 labelling and PTR-MS analysis. *Plant, Cell* and *Environment,* 33: 781–792.

Hammerbacher A, Ralph SG, Bohlmann J, Fenning TM, Gershenzon J, Schmidt A (2011) Biosynthesis of the major tetrahydroxystilbenes in spruce, astringin and isorhapontin, proceeds via resveratrol and is enhanced by fungal infection. *Plant Physiology,* 157: 876–890.

Han JL, Liu BY, Ye HC, Wang H, Li ZQ, Li GF (2006) Effects of over-expression of the endogenous farnesyl diphosphate synthase on the artemisinin content in *Artemisia annua* L. *Journal of Integrative Plant Biology,* 48: 482–487.

Hedden P, Thomas SG (2012) Gibberellin biosynthesis and its regulation. *Biochemical Journal,* 444: 11–25.

Heiling S, Schuman MC, Schoettner M, Mukerjee P, Berger B, Schneider B, Jassbi AR, Baldwin IT (2010) Jasmonate and ppHsystemin regulate key malonylation steps in the biosynthesis of 17-hydroxygeranyllinalool diterpene glycosides, an abundant and effective direct defense against herbivores in *Nicotiana attenuata*. *Plant Cel,l* 22: 273–292.

Hemmerlin A, Harwood JL, Bach TJ (2012) A raison d'être for two distinct pathways in the early steps of plant isoprenoid biosynthesis? *Prog Lipid Res* 51: 95–148.

Hsieh FL, Chang TH, Ko TP, Wang AHJ (2011) Structure and mechanism of an *Arabidopsis* medium/long-chain-length prenyl pyrophosphate synthase. *Plant Physiology,* 155: 1079–1090.

Inoue Y, Hada T, Shiraishi A, Hirose K, Hamashima H, Kobayashi S (2005) Biphasic effects of geranylgeraniol, teprenone, and phytol on the growth of *Staphylococcus aureus*. *Antimicrobial Agents* and *Chemotherapy* 49: 1770–1774.

Ischebeck T, Zbierzak AM, Kanwischer M, Dörmann P (2006) A salvage pathway for phytol metabolism in *Arabidopsis. Journal of Biochemical Biology*, 281: 2470–2477.

Jassbi AR, Gase K, Hettenhausen C, Schmidt A, Baldwin IT (2008) Silencing geranylgeranyl diphosphate synthase in *Nicotiana attenuata* dramatically impairs resistance to tobacco hornworm. *Plant Physiology,* 146: 974–986.

Jondiko IJO, Pattenden G (1989) Terpenoids and an apocarotenoid from seeds of *Bixa orellana*. *Phytochemistry,* 28: 3159–3162.

Kai GY, Xu H, Zhou CC, Liao P, Xiao JB, Luo XQ, You LJ, Zhang L (2011) Metabolic engineering tanshinone biosynthetic pathway in *Salvia miltiorrhiza* hairy root cultures. *Metabolic Engineering,* 13: 319–327.

Kapin MA, Ahmad S (1980) Esterases in larval tissues of gypsy moth, *Lymantria dispar* (L.): optimum assay conditions, quantification and characterization. *Insect Biochemistry,* 10: 331–337.

Karunen P, Mikola H, Ekman R (1980) Occurrence of steryl and wax esters in *Dicranum elongatum*. *Physiologia Plantarum* 48: 554–559.

Keeling CI, Bohlmann J (2006a) Diterpene resin acids in conifers. *Phytochemistry,* 67: 2415–2423.

Keeling CI, Bohlmann J (2006b) Genes, enzymes and chemicals of terpenoid diversity in the constitutive and induced defence of conifers against insects and pathogens. *New Phytologist,* 170: 657–675.

Keena MA (2003) Survival and development of *Lymantria monacha* (Lepi-doptera: Lymantriidae) on North American and introduced Eurasian tree species. *J Economic Entomology,* 96: 43–52.

Keena MA, Vandel A, Pultar O (2010) Phenology of *Lymantria monacha* (Lepidoptera: Lymantriidae) laboratory reared on spruce foliage or a newly developed artificial diet. *Annals of the Entomological Society of America,* 103: 949–955.

Keim V, Manzano D, Fernández FJ, Closa M, Andrade P, Caudepón D, Bortolotti C, Vega MC, Arró M, Ferrer A (2012) Characterization of *Arabidopsis* FPS isozymes and FPS gene expression analysis provide insight into the biosynthesis of isoprenoid precursors in seeds. *PLoS ONE,* 7: e49109.

Kharel Y, Koyama T (2003) Molecular analysis of cis-prenyl chain elongating enzymes. *Natural Product Reports* 20: 111–118.

Klimaszewska K, Lachance D, Pelletier G, Lelu MA, Seguin A (2001) Regeneration of transgenic *Picea glauca*, *P. mariana*, and *P. abies* after cocultivation of embryogenic tissue with *Agrobacterium tumefaciens*. *In Vitro Cell and Developmental Biology Plant,* 37: 748–755.

Klimetzek D, Vite JP (1989) Tierische Schädlinge. In H Schmidt-Vogt, ed, Die Fichte, Vol 2. Verlag Paul Parey, Hamburg, Germany, pp 40–131.

Köksal M, Hu H, Coates RM, Peters RJ, Christianson DW (2011) Structure and mechanism of the diterpene cyclase entcopalyl diphosphate synthase. *Nature Chemical Biology,* 7: 431–433.

Lange BM, Mahmoud SS, Wildung MR, Turner GW, Davis EM, Lange I, Baker RC, Boydston RA, Croteau RB (2011) Improving peppermint essential oil yield and composition by metabolic engineering. *Proceedings of the National Academy of Sciences of the United States of America,* 108: 16944–16949.

Lemaire M, Nagnan P, Clement JL, Lange C, Peru L, Basselier JJ (1990) Geranyllinalool (diterpene alcohol): an insecticidal component of pine wood and termites (Isoptera: Rhinotermitidae) in four European eco-systems. *Journal of Chemical Ecology,* 16: 2067–2079.

Lewinsohn E, Savage TJ, Gijzen M, Croteau R (1993) Simultaneous analysis of monoterpenes and diterpenoids of conifer oleoresin. *Phytochemical Analysis,* 4: 220–225.

Liang PH (2009) Reaction kinetics, catalytic mechanisms, conformational changes, and inhibitor design for prenyltransferases. *Biochemistry,* 48: 6562–6570.

Liang PH, Ko TP, Wang AHJ (2002) Structure, mechanism and function of prenyltransferases. *European Journal of Biochemistry,* 269: 3339–3354.

Liljenberg C (1977) Occurrence of phytolpyrophosphate and acyl esters of phytol in irradiated dark-grown barley seedlings and their possible role in biosynthesis of chlorophyll. *Physiologia Plantarum,* 39: 101–105.

Liljenberg C, Karunen P (1978) Changes in content of phytyl and ger-anylgeranyl esters of germinating *Polytrichum* commune spores. *Physiologia Plantarum,* 44: 369–372.

Lippold F, vom Dorp K, Abraham M, Hölzl G, Wewer V, Yilmaz JL, Lager I, Montandon C, Besagni C, Kessler F, et al (2012) Fatty acid phytyl ester synthesis in chloroplasts of *Arabidopsis*. *Plant Cell,* 24: 2001–2014.

Lütke-Brinkhaus F, Weiss G, Kleinig H (1985) Prenyl lipid formation in spinach chloroplasts and in a cell-free system of *Synechococcus* (cyanobacteria): polyprenols, chlorophylls, and fatty acid prenyl esters. *Planta,* 163: 68–74.

Manzano D, Busquets A, Closa M, Hoyerová K, Schaller H, Kamínek M, Arró M, Ferrer A (2006) Overexpression of farnesyl diphosphate synthase in *Arabidopsis* mitochondria triggers light-dependent lesion formation and alters cytokinin homeostasis. *Plant Molecular Biology,* 61: 195–213.

Manzano D, Fernández-Busquets X, Schaller H, González V, Boronat A, Arró M, Ferrer A (2004) The metabolic imbalance underlying lesion formation in *Arabidopsis thaliana* overexpressing farnesyl diphosphate synthase (isoform 1S) leads to oxidative stress and is triggered by the developmental decline of endogenous HMGR activity. *Planta,* 219: 982–992.

Martin D, Tholl D, Gershenzon J, Bohlmann J (2002) Methyl jasmonate induces traumatic resin ducts, terpenoid resin biosynthesis, and terpenoid accumulation in developing xylem of Norway spruce stems. *Plant Physiology,* 129: 1003–1018.

Martin DM, Gershenzon J, Bohlmann J (2003) Induction of volatile terpene biosynthesis and diurnal emission by methyl jasmonate in foliage of Norway spruce. *Plant Physiology,* 132: 1586–1599.

Masferrer A, Arró M, Manzano D, Schaller H, Fernández-Busquets X, Moncaleán P, Fernández B, Cunillera N, Boronat A, Ferrer A (2002) Overexpression of *Arabidopsis thaliana* farnesyl diphosphate synthase (FPS1S) in transgenic *Arabidopsis* induces a cell death/senescence-like response and reduced cytokinin levels. *Plant Journal,* 30: 123–132.

McKibben GH, Thompson MJ, Parrott WL, Thompson AC, Lusby WR (1985) Identification of feeding stimulants for boll weevils from cotton buds and anthers. *Journal of Chemical Ecology,* 11: 1229–1238.

Monson RK, Jones RT, Rosenstiel TN, Schnitzler JP (2013) Why only some plants emit isoprene. *Plant, Cell and Environment,* 36: 503–516.

Nagel R, Gershenzon J, Schmidt A (2012) Nonradioactive assay for detecting isoprenyl diphosphate synthase activity in crude plant extracts using liquid chromatography coupled with tandem mass spectrometry. *Analytical Biochemistry,* 422: 33–38.

Nogués I, Brilli F, Loreto F (2006) Dimethylallyl diphosphate and geranyl diphosphate pools of plant species characterized by different isoprenoid emissions. *Plant Physiology,* 141: 721–730.

Nualkaew N, Guennewich N, Springob K, Klamrak A, De-Eknamkul W, Kutchan TM (2013) Molecular cloning and catalytic activity of a membrane-bound prenyl diphosphate phosphatase from *Croton stellatopilosus* Ohba. *Phytochemistry*, 91: 140–147.

Pereira AS, Siqueira DS, Elias VO, Simoneit BRT, Cabral JA, Aquino Neto FR (2002) Three series of high molecular weight alkanoates found in Amazonian plants. *Phytochemistry,* 61: 711–719.

Ralph SG, Yueh H, Friedmann M, Aeschliman D, Zeznik JA, Nelson CC, Butterfield YSN, Kirkpatrick R, Liu J, Jones SJM, et al (2006) Conifer defence against insects: microarray gene expression profiling of Sitka spruce (*Picea sitchensis*) induced by mechanical wounding or feeding by spruce budworms (*Choristoneura occidentalis*) or white pine weevils (*Pissodes strobi*) reveals large-scale changes of the host transcriptome. *Plant, Cell and Environment,* 29: 1545–1570.

Reiter B, Lorbeer E (2001) Analysis of the wax ester fraction of olive oil and sunflower oil by gas chromatography and gas chromatography-mass spectrometry. *Journal of the American Oil Chemist's Society,* 78: 881–888.

Rodríguez-Concepción M (2006) Early steps in isoprenoid biosynthesis: multilevel regulation of the supply of common precursors in plant cells. *Phytochemical* Reviews, 5: 1–15.

Sallaud C, Rontein D, Onillon S, Jabès F, Duffé P, Giacalone C, Thoraval S, Escoffier C, Herbette G, Leonhardt N, et al (2009) A novel pathway for sesquiterpene biosynthesis from Z,Z-farnesyl pyrophosphate in the wild tomato *Solanum habrochaites*. *Plant Cell,* 21: 301–317.

Schiebe C, Hammerbacher A, Birgersson G, Witzell J, Brodelius PE, Gershenzon J, Hansson BS, Krokene P, Schlyter F (2012) Inducibility of chemical defenses in Norway spruce bark is correlated with unsuccessful mass attacks by the spruce bark beetle. *Oecologia,* 170: 183–198.

Schilmiller AL, Schauvinhold I, Larson M, Xu R, Charbonneau AL, Schmidt A, Wilkerson C, Last RL, Pichersky E (2009) Monoterpenes in the glandular trichomes of tomato are synthesized from a neryl diphosphate precursor rather than geranyl diphosphate. *Proceedings of the National Academy of Sciences of the United States of America,* 106: 10865–10870.

Schmidt A, Nagel R, Krekling T, Christiansen E, Gershenzon J, Krokene P (2011) Induction of isoprenyl diphosphate synthases, plant hormones and defense signaling genes correlates with traumatic resin duct formation in Norway spruce (*Picea abies*). *Plant Molecular Biology,* 77: 577–590.

Schmidt A, Wächtler B, Temp U, Krekling T, Séguin A, Gershenzon J (2010) A bifunctional geranyl and geranylgeranyl diphosphate synthase is involved in terpene oleoresin formation in *Picea abies*. *Plant Physiology*, 152: 639–655.

Silverstone AL, Chang C, Krol E, Sun TP (1997) Developmental regulation of the gibberellin biosynthetic gene GA1 in *Arabidopsis thaliana. Plant Journal,* 12: 9–19.

Tanaka Y, Hirasawa H (1989) Sequence analysis of polyprenols by 500 MHz 1H-NMR spectroscopy. *Chemistry and Physics of Lipids,* 51: 183–189.

Tanaka Y, Sato H, Kageyu A (1982) Structural characterization of polyprenols by 13C-n.m.r. spectroscopy: signal assignments of polyprenol homologues. *Polymer Journal* (Guildf) 23: 1087–1090.

Vassão DG, Kim SJ, Milhollan JK, Eichinger D, Davin LB, Lewis NG (2007) A pinoresinol-lariciresinol reductase homologue from the creosote bush (*Larrea tridentata*) catalyzes the efficient in vitro conversion of p-coumaryl/coniferyl alcohol esters into the allylphenols chavicol/ eugenol, but not the propenylphenols p-anol/isoeugenol. *Archives of Biochemistry and Biophysics,* 465: 209–218.

Wang KC, Ohnuma S (2000) Isoprenyl diphosphate synthases. *Biochimica et Biophysica Acta* 1529: 33–48.

Wellenstein G, editor (1942) Die Nonne in Ostpreußen (1933–1937): Frei-landstudien der Waldstation für Schädlingsbekämpfung in Jagdhaus Rominten. Monographien zur Angewandten Entomologie, No. 15. Parey, Berlin.

Weng C, Jackson ST (2000) Species differentiation of North American spruce (*Picea*) based on morphological and anatomical characteristics of needles. *Canadian Journal of Botany,* 78: 1367–1383.

Wolff RL, Christie WW, Coakley D (1997) The unusual occurrence of 14-methylhexadecanoic acid in *Pinaceae* seed oils among plants. *Lipids,* 32: 971–973.

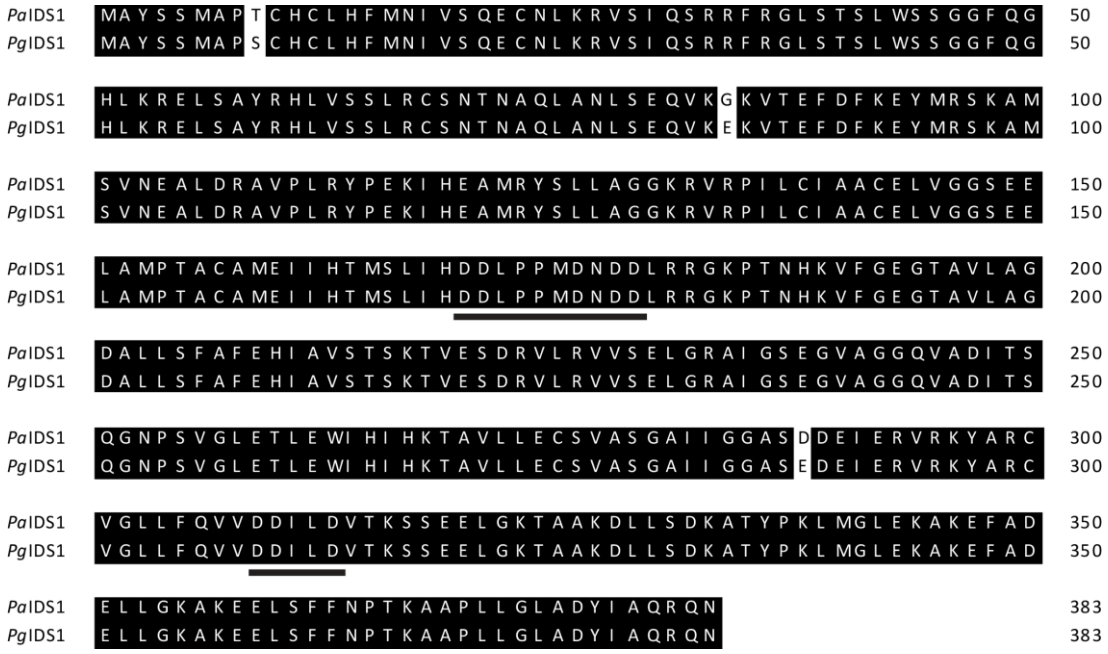
Wolff RL, Lavialle O, Pédrono F, Pasquier E, Deluc LG, Marpeau AM, Aitzetmüller K (2001) Fatty acid composition of *Pinaceae* as taxonomic markers. *Lipids,* 36: 439–451

Wu SQ, Schalk M, Clark A, Miles RB, Coates R, Chappell J (2006) Redirection of cytosolic or plastidic isoprenoid precursors elevates terpene production in plants. *Nature Biotechnology,* 24: 1441–1447.

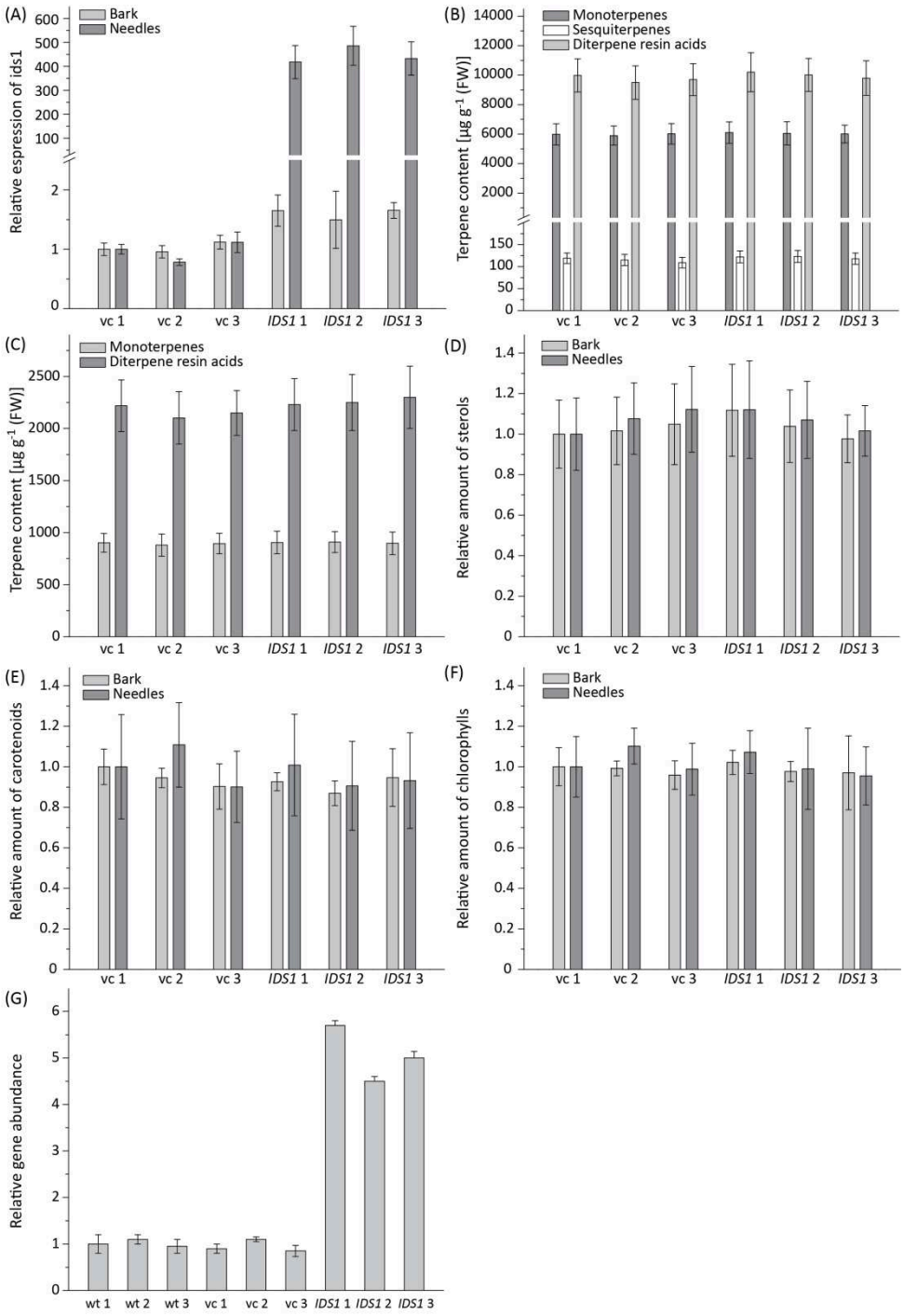
Zhao T, Krokene P, Hu J, Christiansen E, Björklund N, Långström B, Solheim H, Borg-Karlson AK (2011) Induced terpene accumulation in Norway spruce inhibits bark beetle colonization in a dose-dependent manner. *PLoS ONE,* 6: e26649.

Zhou C, Li Z, Wiberley-Bradford AE, Weise SE, Sharkey TD (2013) Isopentenyl diphosphate and dimethylallyl diphosphate/isopentenyl di-phosphate ratio measured with recombinant isopentenyl diphosphate isomerase and isoprene synthase. *Analytical Biochemistry,* 440: 130–136.

2.8. SUPPLEMENTAL DATA

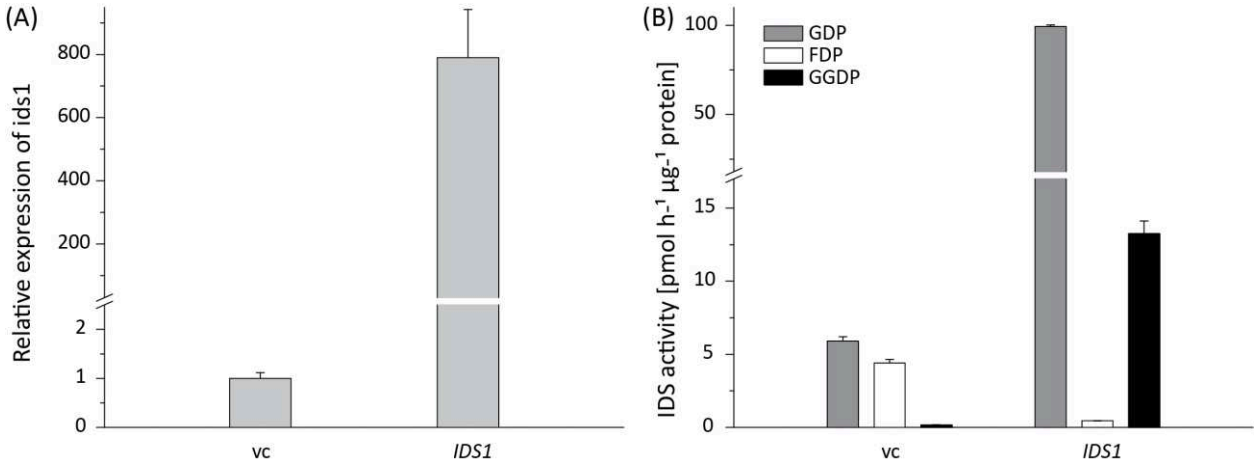


Supplemental Figure 1: Alignment of deduced amino acid sequences of *Pa*IDS1 (accession no. GQ369788) and *Pg*IDS1 (accession no. KF840686). Identical amino acids are boxed in black. The Asp-rich motifs conserved among IDSs are indicated by the black lines.

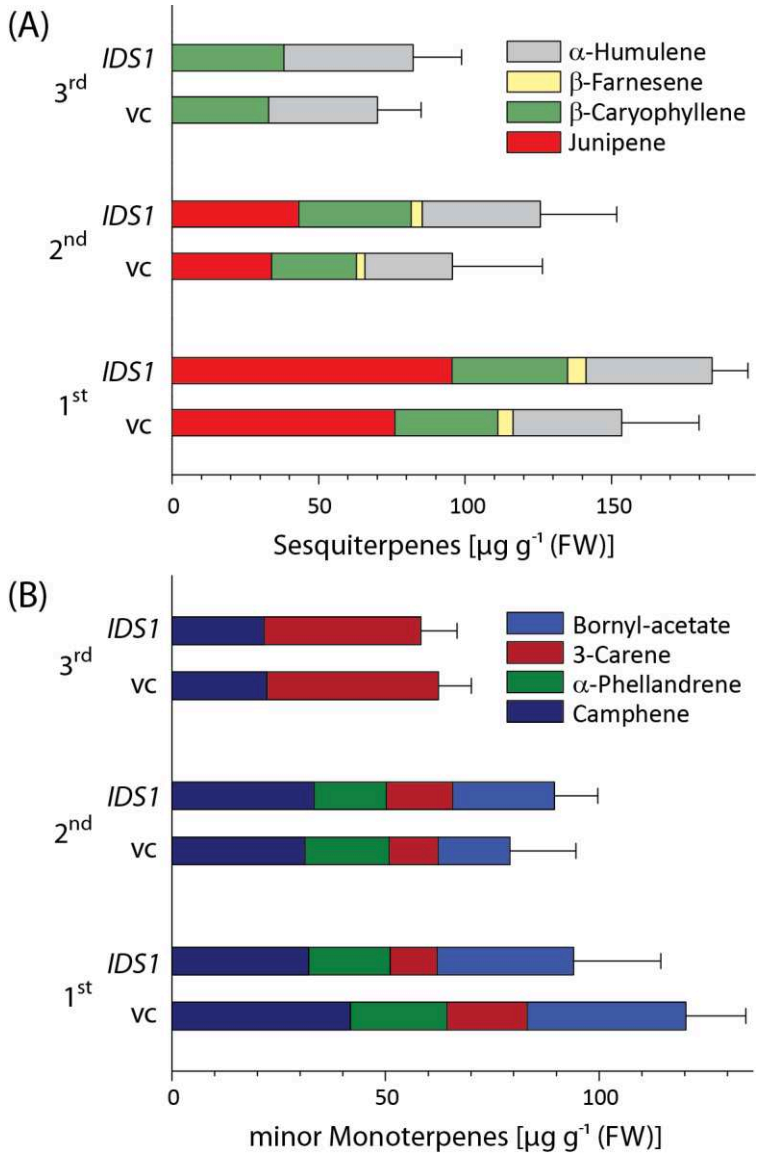


Supplemental Figure 2: Initial characterization of transgenic *P. glauca* saplings overexpressing *IDS1* (*IDS1*) compared with those transformed with an empty vector as a control (vc). Data for three independently-transformed lines of each type are shown. (A) Relative expression of *IDS1* in bark and needles as determined by qPCR. (B) Terpene content of bark (C) Terpene content of needles (D) Sterol content of bark and needles as measured by GC-FID. (E) Carotenoid and (F) chlorophyll content of bark and needles as measured by HPLC-UV/DAD. (G) Relative gene abundance as determined by genomic-qPCR. Data are means ± SD of four plants of each line.

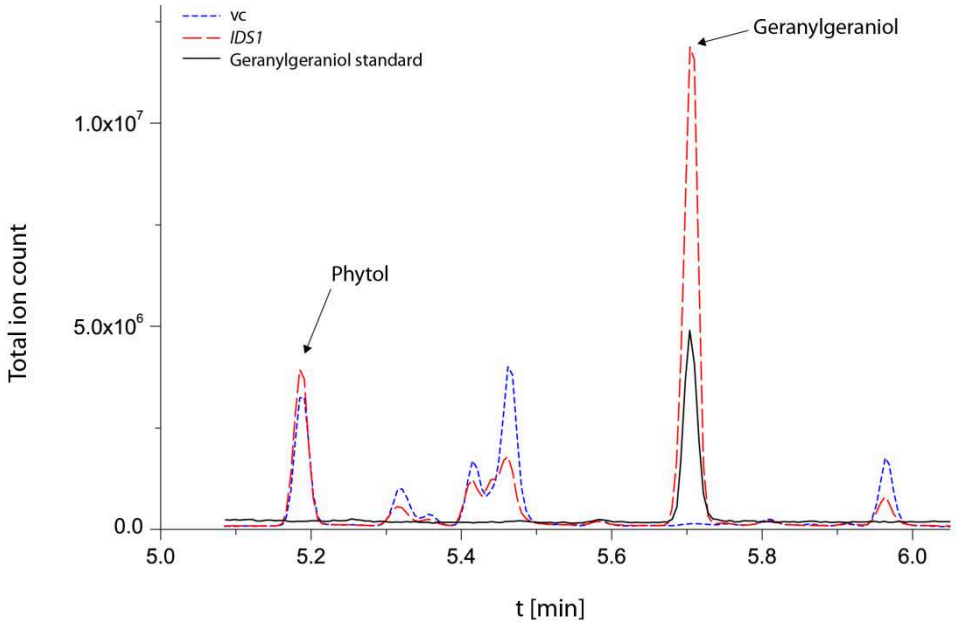
Supplemental Figure 3: Morphology of three-year-old *P. glauca* saplings transformed with (A) empty vector as control or (B) an *IDS1*-overexpressing construct.



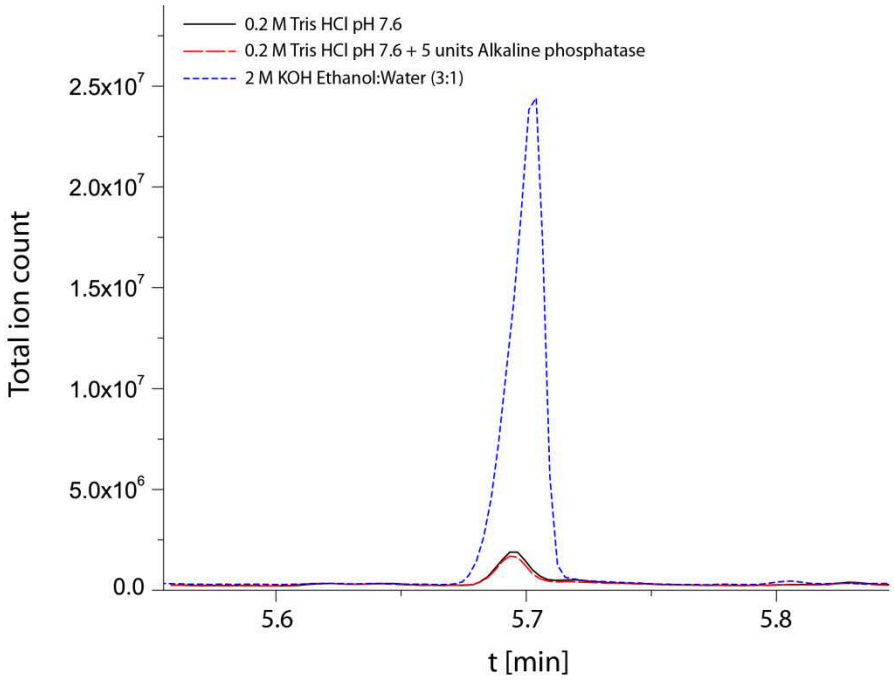
Supplemental Figure 4: Comparison of side branch needles of a *P. glauca IDS1*-overexpressing line (*IDS1*) and an empty vector control line (vc) for (A) relative expression of *IDS1* as determined by qPCR and (B) total IDS enzyme activity as measured in vitro by LC-MS/MS. Data are means ± SD of measurements from five plants of each line.



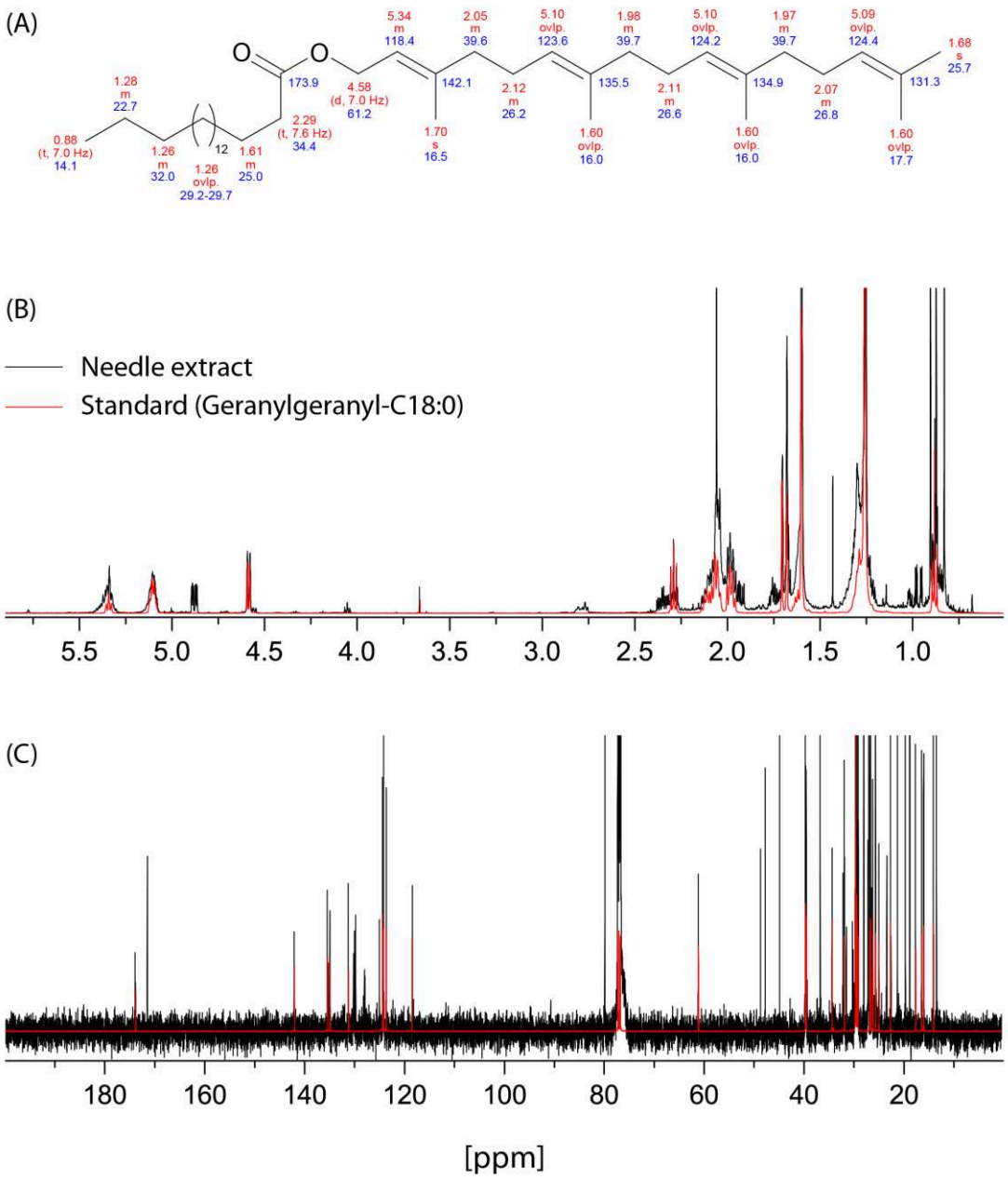
Supplemental Figure 5: Comparison of bark (A) sesquiterpene and (B) minor monoterpene content as measured by GC-FID between three-year-old *P. glauca IDS1*-overexpressing saplings (*IDS1*) and vector control (vc) saplings in 1st, 2nd and 3rd year growth. Data are means ± SD of measurements from five plants of each line.



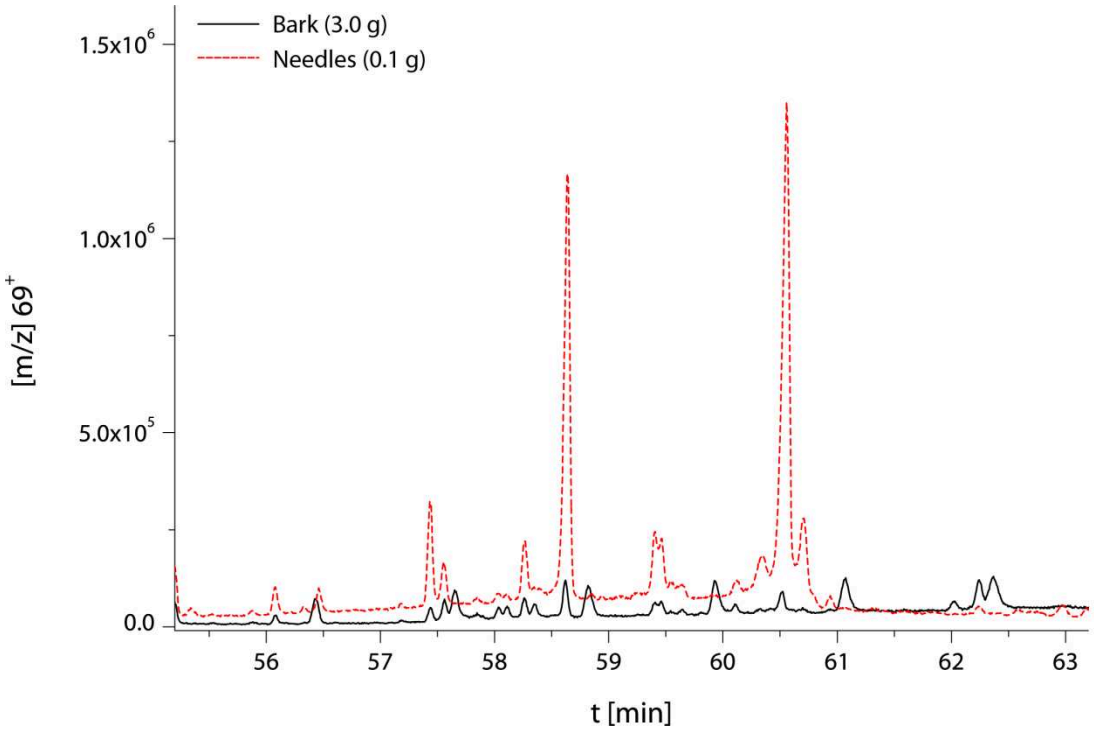
Supplemental Figure 6: GC-MS chromatogram of saponified and silylated lipid extract of either vector control or *IDS1*-overexpressing plants, as well as a silylated authentic geranylgeraniol standard.



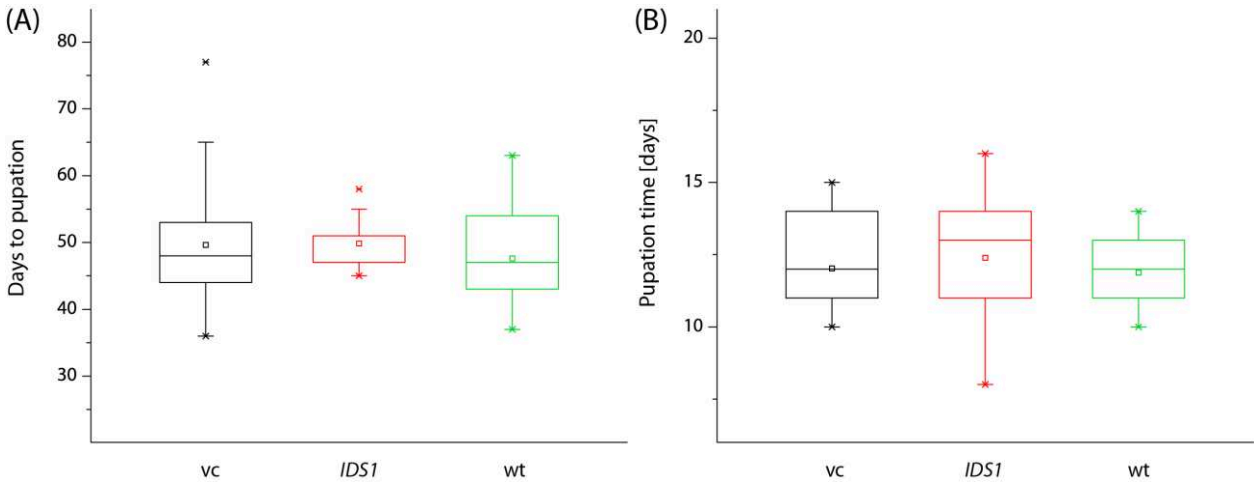
Supplemental Figure 7: Characterization of geranylgeranyl fatty acid esters from *IDS1*-overexpressing *P. glauca* lines. Hydrolysis was attempted in strong base and with alkaline phosphatase, but was only successful in the former case, suggesting compounds were carboxylate esters and not phosphate esters. Peak at 5.7 min is that of geranylgeraniol.



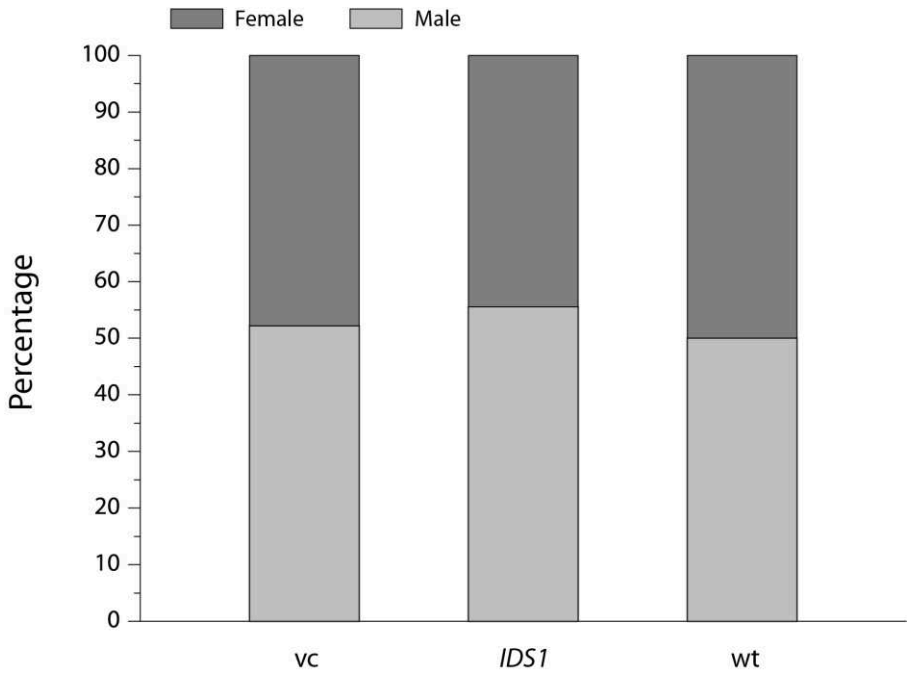
Supplemental Figure 8: NMR analysis of geranylgeranyl fatty acid esters in needle extracts of *IDS1*-overexpressing *P. glauca* plants. (A) Chemical shifts of synthesized geranylgeranyl-C18:0, ^{13}C in blue and ^{1}H in red. (B) ^{1}H-NMR and (C) ^{13}C-NMR spectra of needle extract from *IDS1*-overexpressing saplings overlaid on spectrum of geranylgeranyl-C18:0 standard.



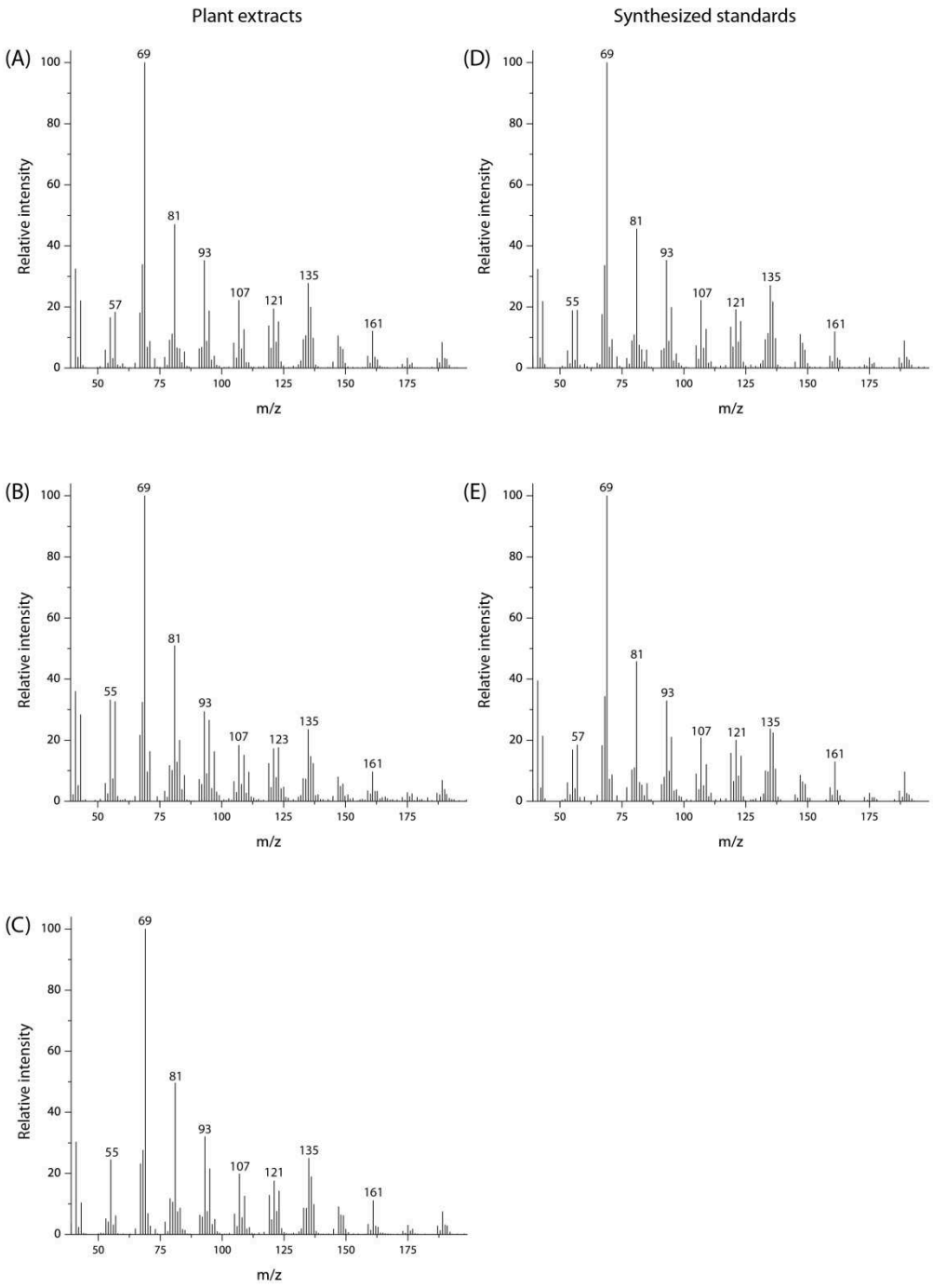
Supplemental Figure 9: Low abundance of geranylgeranyl fatty acid esters in bark vs. needles of *IDS1*-overexpressing *P. glauca* saplings is shown by GC-MS comparison of extracts. Bark extract is 30-fold more concentrated than needle extract, but gives only small peaks. Major peak at ~58.6 min is that of geranylgeranyl–C16:0 and peak at ~60.6 min is that of geranylgeranyl-C18:1 and C18:2.



Supplemental Figure 10: Effect of geranylgeranyl fatty acid esters on development of *Lymantria monacha* larvae feeding on *IDS1*-overexpressing (*IDS1*), empty vector control (vc) and wild-type control (wt) saplings. (A) Time from hatching to pupation and (B) time from pupation to emergence of adult moths.



Supplemental Figure 11: Effect of geranylgeranyl fatty acid esters on sex ratio of emerging *Lymantria monacha* adults after larval feeding on *IDS1*-overexpressing (*IDS1*), empty vector control (vc) and wild-type control (wt) saplings.



Supplemental Figure 12: EI-MS spectra of geranylgeranyl fatty acid esters. (A) geranylgeranyl-C16:0; (B) geranylgeranyl-aiC17:0; (C) mixture of geranylgeranyl-C18:1 and geranylgeranyl-C18:2; (D) geranylgeranyl-C17:0; (E) geranylgeranyl-C18:0. A-C were isolated from plant extracts and D and E were synthesized as standards.

CHAPTER III

THE GUT MICROBIOTA OF THE PINE WEEVIL IS SIMILAR ACROSS EUROPE AND RESEMBLES THAT OF OTHER CONIFER-FEEDING BEETLES

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3.1. ABSTRACT

The pine weevil (*Hylobius abietis*, Coleoptera: Curculionidae) is an important pest of conifer seedlings in Europe. Despite its economic importance, little is known about the composition of its gut microbial community and the role it plays in mediating the weevil's ability to utilize conifers as a food source. Here, we characterized the gut bacterial communities of different populations of *H. abietis* across Europe and compared them to those of other beetles that occupy similar ecological niches. We demonstrate that the microbial community of *H. abietis* is similar at higher taxonomic levels (family and genus) across locations in Europe, with *Wolbachia* as the dominant microbe, followed by Enterobacteria and Firmicutes. Despite this similarity, we observed consistent differences between countries and locations, but not sexes. Our meta-analysis demonstrates that the gut bacterial community of the pine weevil is very similar to that of bark beetles that also exploit conifers as a food source. The Enterobacteriaceae symbionts of both host taxa are especially closely related phylogenetically. Conversely, the microbiota of *H. abietis* is distinct from that of closely related weevils feeding on non-conifer food sources, suggesting that the microbial community of the pine weevil is determined by the environment and may be relevant to host ecology. Furthermore, several *H. abietis*-associated members of the Enterobacteriaceae family are known to contain genes involved in terpenoid degradation. As such, we hypothesize that the gut microbial community is important for the utilization of conifer seedlings as a food source, either through the detoxification of plant secondary metabolites or through the supplementation of essential nutrients.

3.2. INTRODUCTION

Conifers represent a challenging resource for herbivorous insects. These trees contain high amounts of both constitutive and inducible chemical defense compounds, such as phenolics and terpenoids (Keeling and Bohlmann 2006; Li *et al.* 2012) that are toxic or deterrent to herbivores. In addition, many parts such as bark and wood have a high C:N ratio and are poor in essential amino acids, phosphorous, vitamins and sterols (Thornber and North-cote 1961a,b, 1962; Warren and Adams 2002).

Despite the poor nutritional value of conifer tissues, many insects across different orders (e.g. Lepidoptera, Coleoptera, Hemiptera) are able to exploit this food source using different mechanisms. Most of the research effort has so far focused on bark beetles (Coleoptera: Curculionidae: Scolytinae), due to their economic and ecological importance. For example, the bark beetle *Ips grandicollis* overcomes the poor nutritional quality of conifers by increasing its phloem consumption rate compared to that of mycangial bark beetles (i.e. those that harbor symbiotic fungi in a cavity called mycangium) (Ayres *et al.* 2000). In some other bark beetles, cooperative behavior can mitigate the effects of conifer defenses. For example, the larvae of *Dendroctonus micans, Dendroctonus terebrans* and *Dendroctonus valens* feed in cavities under the bark as groups in one continuous front, thus probably outrunning the tree-induced chemical defenses (Gregoire *et al.* 1981; Deneubourg *et al.* 1990). Other bark beetle species colonize healthy trees by attacking in large numbers, thereby exhausting the tree defenses and ultimately killing their host (Berryman 1976). Sawflies (Diprionidae) damage the resin ducts of their conifer host prior to feeding, to release part of the chemical defenses, or feed gregariously to consume the needles before defenses are induced (McCullough and Wagner 1993). These insects can also sequester conifer resins and use them against predators with an efficiency that is correlated with the host tree chemotype (Codella and Raffa 1995).

While some herbivores rely on their own capabilities to cope with the low nutrient content and toxic defenses of their host plants, others engage in symbiotic relationships with microbes that supplement limiting nutrients or aid in degradation of toxic compounds (Douglas 2009). For conifer-feeding insects, most of the available information on symbionts concerns fungi that provide nutritional benefits or detoxify plant secondary metabolites. Three different bark beetle species (*Drendoctonus frontalis, Drendoctonus ponderosae* and *Drendoctonus brevicomis*) harbor symbiotic fungi in their mycangia that supplement the insect diet with assimilated nitrogen (Six and Paine 1998; Ayres *et al.* 2000; Bleiker and Six 2007). Specifically, after inoculation into the host tree by the beetle, the fungi assimilate sapwood nitrogen and transport it to the bark and phloem, where the beetle larvae feed, thereby increasing the available nitrogen content by as much as 40% (Bleiker and Six 2007). In addition, *D. ponderosae* acquires sterols from ophiostomatoid fungi during the larval stage, which are important for the beetle's fecundity (Six and Paine 1998; Bentz and Six 2006). Lack of vitamins in coniferous trees is overcome by bark beetles through association with symbiotic yeasts (Strongman 1987; Pignal *et al.* 1988). Furthermore, symbiotic fungi are also involved in insect resistance to conifer chemical defenses. *Grossmania clavigera*, a bark beetle symbiont, is able to cope with terpenoids by mediating their transport outside the fungal cell via ABC transporters (Wang *et al.* 2013), and it can use monoterpenes as a carbon source (DiGuistini *et al.* 2011). Additionally, putative detoxification genes including O-methyltransferases and CYP450s are upregulated in *G. clavigera* following exposure to terpenoids (DiGuistini *et al.* 2011), with O-methyltransferases known to degrade phenolic compounds (Feltrer *et al.* 2010), and CYP450s to detoxify a number of plant secondary metabolites (Wöll *et al.* 2013). In comparison with fungal symbionts, the contributions of bacterial associates towards conifer-feeding in insects remain poorly known (Grossmann 1930; Craighead and George 1940; Barras 1967; Whitney and Cobb 1972; Paine *et al.* 1997). Gut bacteria in *D. ponderosae* beetles have been found to supplement their host's diet with nitrogen (Morales-Jimenez *et al.* 2009), bacteria isolated from the gut of *Dendroctonus rhizophagous* showed cellulolytic activity on

plate (Morales-Jimenez *et al.* 2012), and gut bacteria from *D. valens* were able to degrade mono- and diterpenes in vitro (Boone *et al.* 2013). However, how this affects the insects' fitness remains to be determined. Likewise, the mechanistic basis of the degradation of these compounds remains unclear, although a complete diterpene degradation gene cluster (DDGC) has been found in the bacterial metagenome of *D. ponderosae* (*Adams et al.* 2013).

The pine weevil, *Hylobius abietis* (Coleptera: Curculionidae: Molytinae), feeds primarily on the phloem tissue of several conifers (mainly Scots pine, *Pinus sylvestris*, and Norway spruce, *Picea abies*), where the bark is thin. In some instances, especially when feeding on the stem bark of newly planted conifer seedlings, the pine weevil can cause over 80% mortality (Petersson and Orlander 2003). Thus, *H. abietis* is considered the most important pest of European conifer forests that are managed by clear-cutting followed by replanting (Leather *et al.* 1999; Nordlander *et al.* 2011). Because of its economic importance, the biology and ecology of the pine weevil have received considerable attention over the last decades (Wallertz *et al.* 2006; Wainhouse *et al.* 2014). In spring, after hibernation in the soil, adult pine weevils disperse by flight and may migrate very long distances (up to about 100 km) in their search for suitable reproduction sites (Solbreck 1980). In managed forest landscapes, pine weevils land mainly on newly clear-cut areas, to which they are attracted by volatiles released from fresh conifer stumps (Solbreck and Gyldberg 1979). During summer, weevils remain on the clear-cut where they feed and mate, and females lay their eggs in the bark of stump roots or in the soil nearby (Nordlander *et al.* 1997). The larvae feed underground, tunneling in the bark of the stump roots, whereas adults feed on conifer bark both below and above the surface of the soil (Nordlander *et al.* 2005; Wallertz *et al.* 2006). Thus, pine weevils are closely associated with forest soil and conifer bark, encountering high concentrations of terpenoids in their diet throughout their life. As these compounds are toxic to a wide range of insects (Keeling and Bohlmann 2006), it seems imperative that the weevil must have evolved mechanisms to detoxify or tolerate them. However, whether they do this on their own or in association with symbiotic microbes remains unknown.

In this study, we investigated whether *H. abietis* harbors a consistent microbial community across distant geographical locations and explored whether symbiotic microbes may be involved in terpenoid detoxification. To this aim, we characterized the bacterial gut community of pine weevils from six different locations across Europe using 454 pyrosequencing of bacterial 16S rRNA amplicons and oligotyping, to assess their composition and overall stability. To gain first insights into possible roles of the microbiota in pine weevil host ecology, we predicted the bacterial metagenome function based on the 16S rRNA gene, and we performed a meta-analysis to compare the gut bacterial community of beetle species feeding on conifer- vs. non-conifer-based food sources. Due to the importance of bacterial symbionts for digestive processes of insects, we expect beetle species feeding on similar (coniferous) food sources to exhibit convergence in their microbial community structure. Our results provide an ecological perspective on gut microbial community composition in conifer-feeding beetles, highlighting the potential role of microbial symbionts in exploitation of conifers as a food source.

3.3. MATERIALS AND METHODS

3.3.1. Insect collection and sample preparation

Pine weevils were collected from fresh clear cuts in different locations in Europe (Fig. S1, Supporting information). We sampled six locations following a North–South transect: Umea, Uppsala, Asa and the isolated island of Gotland in the Baltic Sea are all located in Sweden, while locations in Hannover in

Germany and Galicia in Spain were also sampled. Weevils were captured alive with clean pitfall traps baited with a-pinene and ethanol (Nordlander 1987). Vials without lids were filled with ethanol, and the entrance was blocked with bait-impregnated paper towels or cotton and placed leaning towards a pine branch. Once collected, the weevils were placed in boxes with holes for ventilation. They were sent to the laboratory in Styrofoam boxes with ice packs wrapped with paper to avoid insect freezing.

3.3.2. DNA extraction, amplification and sequencing

Insects were dissected using dissecting scopes under sterile conditions. Only guts from insects free of apparent nematode infections were used for further analyses. An average of five isolated guts from weevils caught in the same trap were pooled (Table S1, Supporting information). Isolated guts were placed in Eppendorf tubes, flash frozen with liquid nitrogen and homogenized with a pestle. Then, samples were pretreated with 180 μl of enzymatic lysis buffer for Gram-positive bacteria (20 mM Tris-Cl, pH 8.0; 2 mM sodium EDTA, 1.2% Tri-ton X-100 and addition of lysozyme to 20 mg/ml). Total DNA was extracted by a Qiacube automated extraction robot (Qiagen) using the QIAamp DNA mini kit (Qiagen).

Emulsion PCR was performed with PCR beads (0.2-ml PuReTaq Ready-To-Go PCR beads; GE Healthcare) and sample-identifying tags, known as Multiplex Identifier Adaptors for Rapid Library Preparations (Technical Bulletin No. 2010-010; Roche) (Swanson *et al.* 2011) connected to the general eubacterial primers 8f (50-AGAGTTTGATIITGGCTCAG-30) and 1501r (50-CGGI-TACCTTGTTACGAC-30) (Lindh *et al.* 2005), targeting the V1–V9 region of the 16S rRNA gene. The DNA amplification program was as follows: 94 °C for 3 min, followed by 30 cycles of 94 °C for 30 s, 58–48 °C for 30 s (the temperature was decreased by 1 °C every cycle for 10 cycles and then held at 48 °C for 20 cycles) and 72 °C for 1 min 30 s, followed by a final extension step at 72 °C for 25 min (Lindh *et al.* 2005). Size and quantity of PCR products were determined by a MultiNA Microchip Electrophoresis System (Shimadzu). Amplicons from each sample were diluted in equimolar amounts and sequenced on a 454-FLX system using Titanium chemistry (454 Life Sciences, Brandford, CT) at the SNP/SEQ platform hosted by SciLife Lab, Uppsala, Sweden.

3.3.3. 454 sequencing analysis

Processing of high-quality reads was performed using QIIME (Caporaso *et al.* 2010). We retained sequences between 200 and 600 bp in length, allowing no errors in the barcode but one mismatch in the primer and one ambiguous base. The minimum average quality score per read was set to 25, and reads that were not assigned to any barcode were discarded. Potential chimeric sequences were identified with USEARCH61 (QIIME) and removed from the data set. The resulting sequences were subjected to both open and closed-reference operational taxonomic unit picking strategies. Open-reference OTU picking was performed with the algorithm cdhit (QIIME) using 97% similarity as a threshold to cluster the sequences into OTUs. The most abundant sequence for each OTU was selected as a representative sequence. Taxonomy was assigned using RDP classifier (Wang *et al.* 2007), and remaining sequences with <0.8 confidence in their assignment were removed. Closed-reference OTU picking was also performed at 97% similarity against the GREENGENES database released in May 2013 (http://greengenes.lbl.-gov/). OTU tables were generated describing the abundance of bacterial phylotypes within each sample. Results for both methods were compared after manually filtering chloroplast and mitochondrial sequences as well as singletons.

For downstream analyses, the raw OTU table obtained after open-reference OTU picking strategy was used. The OTU table was manually filtered, that is chloroplast and mitochondrial sequences and OTUs with <0.1% abundance in all samples were removed, and samples with <500 reads were eliminated. As the high abundance of *Wolbachia* overshadowed the under-lying microbial gut community in most samples, we removed *Wolbachia* reads after initial analysis. Samples with <400 reads were eliminated,

and OTU tables were rarified to the minimum depth of 481 reads per sample. Representative sequences were blasted against the NCBI database to identify their top hit using BLAST2GO (Conesa *et al.* 2005) to confirm taxonomic assignment. OTU abundance within samples was visualized in a heat map constructed with the MULTIEXPERIMENT VIEWER (MEV) software (Saeed *et al.* 2003).

Alpha-diversity estimates such as observed species richness and Chao1 (reported for 3% difference between sequences) were calculated. Rarefaction curves were obtained in QIIME by subsampling the OTU table with step increments of 10 sequences and 100 iterations at each step to see the adequacy of our sampling. Beta-diversity metrics (including abundance-weighted and unweighted UniFrac distances, binary Jaccard and Bray–Curtis dissimilarities, and Sorensen) were calculated using the same OTU table as above. The pylogenetic tree needed for beta-diversity analyses was produced with FASTTREE (Price *et al.* 2009) by aligning the representative sequences to the Greengenes core set (http://greengenes.lbl.gov/) using PYNAST, with a minimum sequence identity of 75%. All beta-diversity metrics and principal coordinate analysis (PCoA) plots were generated in QIIME. We statistically tested whether the association of OTUs with the weevils was dependent on site and sex, respectively, by independently analyzing the matrixes with one-way analyses of similarities (ANOSIM) and Adonis. In parallel, we performed discriminant analyses (DA) in SPSS 17.0 on the OTU table containing the whole community, and also on an OTU table containing only the 15 most abundant OTUs. In both cases, we performed a principal component analysis (PCA) prior to the DA and retained only the first four principal components, in order to avoid excessive numbers of variables in the DA.

We also used QIIME to calculate the 'core' microbiome (using the script core_microbiome.py), defined as the OTUs present in ≥50% of all samples, for samples belonging to each location and each country. We visualized OTUs shared by the three countries using a Venn diagram with VENNY online software (Oliveros 2007).

3.3.4. Strain diversity analysis by oligotyping

To study strain diversity within the members of the most prevalent taxa, we carried out an oligotyping analysis (version 2.0) (Eren *et al.* 2013). We extracted all sequences belonging to the taxa of interest and kept sequences of 350 bp of length, excluding all sequences that were shorter. Sequences were aligned with PYNAST against the GREENGENE database (http://greengenes. lbl.gov/), and uninformative gaps were eliminated with the script o-trim-uninformative-columns-from-alignment (http://github.com/meren/oligotyping/blob/master/bin/). The ends of the sequences were trimmed to a common length with the script o-trim (same source as above). After the initial entropy analyses with the entropy-analysis script of the oligotyping pipeline, we ran oligotyping, selecting as many high entropy positions as necessary for oligotypes to converge. We considered an oligotype to have converged when all nucleotide positions had entropy values below 0.2. To reduce noise, each oligotype was required to have a minimum of 40 copies of the most abundant unique sequence. Oligotypes that did not meet this criterion were excluded from the analysis. We statistically tested whether the distribution of the different oligotypes was associated with geography or sex by calculating a Bray– Curtis dissimilarity matrix on the oligotyping output table and analyzing the matrix with ANOSIM.

3.3.5. Functional inference on the bacterial metagenome

We used Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUST, version 1.0.0) to predict metagenome function based on 16S rRNA sequences using the GREENGENES database of reference genomes (Langille *et al.* 2013). For this purpose, we used the OTU table obtained after closed-reference OTU picking strategy. We rarefied the OTU table to 497 reads, and OTUs were normalized by 16S rRNA gene copy number. The predicted abundance of different gene families was

calculated using Kyoto Encyclopaedia of Genes and Genomes (KEGG; Kanehisa *et al.* 2012). Nearest Sequenced Taxon Index (NSTI) was calculated to assess the average similarity between the sequences of an OTU and those of the nearest sequenced genome present in the database. This value gives estimation on the accuracy of the PICRUST analysis. We used the PICRUST output table to build a heat map with MEV (Saeed *et al.* 2003).

The KEGG database does not include some genes of interest for this study, particularly diterpene degradation genes. Therefore, we additionally searched homologues of the DDGC described in Smith *et al.* 2004 using BLASTP (2.2.25+) in the NCBI NR database (downloaded in February 2014). The cut-off for BLAST was E-value ≤1e 5. The species ID of all identified DDGC protein homologues were retrieved from NCBI using TAXA database. Subsequently, they were compared to the identified taxa from our representative set. Only OTUs with genus-level taxonomic assignment that matched one of the DDGC-containing species from NCBI were scored as positive for DDGC. Given that some OTUs (especially those within the Enterobacteria) in our study were not taxonomically assigned beyond family, we performed a second homologue search against a custom database. This database comprised fully sequenced genomes of close relatives to the pine weevil0s core microbiota. The cut-off for BLAST was E-value ≤1e 5.

3.3.6. Meta-analysis: phylogenetic placement of *Hylobius abietis* gut microbiota

To interpret the pine weevil microbial community com-position in an ecological context, we tested whether it was consistent with that of other conifer-feeding beetles. Our data set on *H. abietis*-associated bacteria was expanded by the addition of 308 bacterial sequences from GenBank associated with 10 different insect host species belonging to four genera. These sequences included bacterial taxa found in the gut microbiota of five species of bark beetles belonging to two genera that feed on conifers, as well as five weevil species belonging to two genera that feed on red palm trees and ornamental plants, respectively (Table S2, Supporting information). We only selected culture-independent studies. Database sequences were aligned with those obtained in this study using SINA (Pruesse *et al.* 2012). The alignment was imported into ARB (Ludwig *et al.* 2004) together with the SILVA 115 database (http://arb-silva.de/projects/living-tree/) and a phylogenetic tree was constructed. We refined the alignment by selecting all *H. abietis*-associated sequences and those of closely related bacterial type strains present in the SILVA database. Due to the large number of sequences that were selected, the final alignment was produced by filtering some sequences: we only retained those sequences from the pine weevil that corresponded to the 50 most prevalent OTUs (i.e. those that are more often found across samples) and, in addition, those of minor OTUs that clustered together with other bark beetle or weevil-associated bacterial sequences. We also eliminated those bacterial sequences from bark beetles and weevils (other than *H. abietis*) that did not cluster together with any other insect-associated bacteria in this study. The final phylogeny was reconstructed with FASTTREE using the GTR model (Price *et al.* 2010) and edited in MEGA (Tamura *et al.* 2011).

3.4. RESULTS

3.4.1. The microbial community of *Hylobius abietis* guts

The gut bacterial community of the pine weevil was characterized using tag-encoded FLX amplicon pyrosequencing of the bacterial 16S rRNA gene. A total of 234 055 high-quality sequences were generated across 51 samples from three different countries. The sequences were binned to 359 OTUs, of which 96 were removed because they were of likely chimeric origin. Both raw OTU tables obtained with

open and closed-reference OTU picking methods were very similar, containing 263 and 257 OTUs, respectively. After filtering singletons as well as chloroplast and mitochondrial sequences, each OTU table contained 249 and 255 for open- and closed-reference methods, respectively. Taxonomical assignment was similar regardless of the OTU picking strategy used (Fig. S2 and S3, Supporting information).

We used the OTU table obtained with open-reference methods for downstream analyses. Two OTUs were removed because they were classified as chloroplasts and another two because they belonged to samples with <500 reads. The remaining 259 OTUs contained 231 706 sequences, representing 98.99% of all input sequences (Table S1, Supporting information).

At high taxonomic levels, the bacterial community found in the weevil's gut was quite stable, dominated by Alpha- and Gamma-Proteobacteria in 90% of the samples (Fig. 1). Firmicutes were abundant in Germany and some samples from Gotland (Sweden), but were absent from Spanish samples. Swedish samples harbored a higher bacterial diversity than samples from Germany or Spain, with low abundances of Actinobacteria and other taxa (Fig. 1). Most alpha-proteobacterial sequences were assigned to *Wolbachia*, which was present in all samples, ranging, in relative abundance, from 0.2% to 100% and comprising on average 73%, 28% and 49% of sequence reads in samples from Sweden, Germany and Spain, respectively. However, there was no statistical difference in the relative abundance of *Wolbachia* among countries, locations or sexes (Kruskal–Wallis test, d.f. = 2; d.f. = 5; d.f. = 1, P = 0.4; P = 0.2, and P = 0.1, respectively).

Due to its high abundance, *Wolbachia* overshadowed the remaining microbial community. Therefore, we eliminated *Wolbachia* sequences from the analysis to gain more detailed insights into other microbial associates. Following the removal of *Wolbachia*, 23 samples had <400 reads and were excluded from further analysis. To normalize the number of reads per sample, we rarefied the OTU table to a common depth of 481 reads per sample. Our final *Wolbachia*-free data set contained 13 468 reads that were binned to 162 OTUs distributed across 28 samples (Table S1, Supporting information).

Rarefaction analysis indicated that despite removing *Wolbachia* reads, our sampling of the underlying microbial community was still exhaustive (Fig. S4, Supporting information). Calculation of the Chao1 diversity index revealed considerable variability between samples, ranging from 6 to 48 OTUs, with the mean SD being 16 8 OTUs (Fig. S4, Supporting information). However, there were no differences in richness between countries (Fig. S5, Supporting information).

After removing *Wolbachia* sequences, the most abundant and prevalent OTUs belonged to the families Enterobacteriaceae (Gamma-Proteobacteria) and Leuconostocaceae (Firmicutes) (Fig. 2). Within these two families, two patterns emerged. First, within the Enterobacteriaceae, *Erwinia* sp. was very abundant across Spanish and German samples, but virtually absent from all locations in Sweden. Second, within the Leuconostocaceae, *Weissella oryzae*, which was the most abundant taxon within the family, and *Lactococcus* sp., were present in Swedish and German samples, but completely absent from Spanish ones. Interestingly, some samples were dominated by Proteobacteria and others by Firmicutes, with no sample harbouring similar relative abundances of these two taxa (Fig. 2).

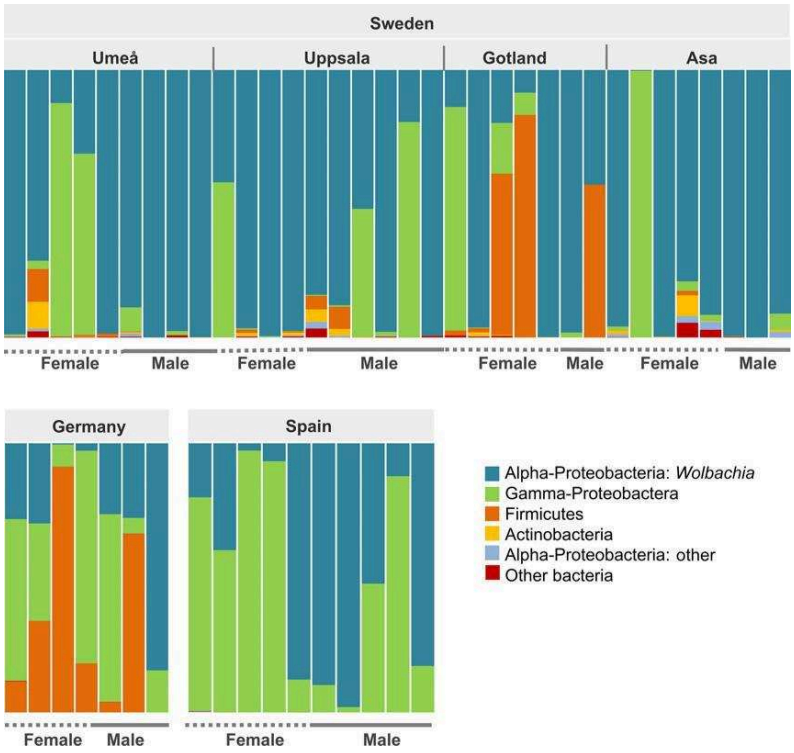

Fig. 1 Class-level gut bacterial community composition of *Hylobius abietis* across different geographical locations. Each bar corresponds to a pool of three to nine individuals of the same sex collected from the same trap.

As expected based on the higher diversity, most OTUs (134) were present in Sweden, with 100 of those being exclusive, while only 51 and 38 were found in Germany and Spain, respectively (Fig. S6, Supporting information). Only 16 OTUs—13 Enterobacteriaceae and three Firmicutes—were shared between samples from all three countries. The analysis of the 'core' microbiota (i.e. those OTUs present in more than 50% of the samples) revealed just four OTUs, all of them belonging to the Enterobacteriaceae family, which were shared by all three countries. While genus-level classifications are generally difficult in the Enterobacteriaceae based on short 16S rRNA fragments, BLAST analyses of the 'core' microbes suggested that their closest relatives are *Erwinia typographi, Rahnella* and *Serratia*.

From the 50 most prevalent OTUs (i.e. those that were found in most samples) that comprised 97.2% of the original sequences, 31 belonged to the Gamma-Proteobacteria and two to the Alpha-Proteobacteria. Within the Gamma-Proteobacteria, all OTUs except for one Pseudomonadales OTU belonged to the Enterobacteriaceae family. The remaining 17 OTUs comprised 12 Firmicutes and five Actinobacteria (Fig. 2).

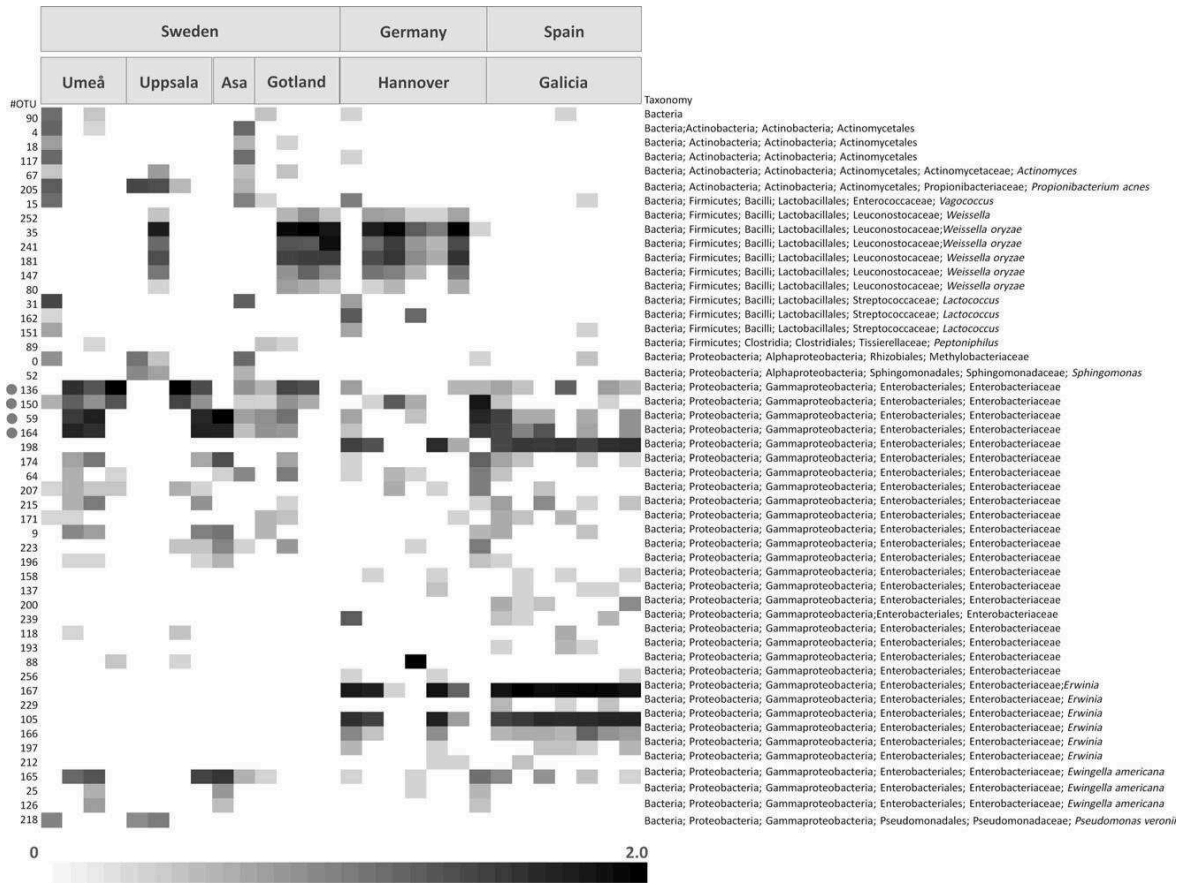

Fig. 2 Bacterial gut community composition of the pine weevil from different geographical locations after removal of *Wolbachia*-associated OTUs. Only the 50 most prevalent OTUs are shown. Relative abundances of bacterial taxa are displayed as a heat map on log scale, with dark colors representing high abundances and white indicating absence.

3.4.2. Variation in microbial communities due to geography

We used several different distance metrics to assess differences in bacterial community profiles between sexes and geographical locations. PCoA based on an unweighted UniFrac distance matrix (Fig. 3) and DA revealed that the gut microbiota composition of weevils does not differ between sexes (ANOSIM P = 0.623; Adonis P = 0,773; Wilk0s k = 0.88 1, P = 0.546). However, the composition significantly differed between the three countries tested (ANOSIM P = 0.001; Adonis P = 0.006; Wilk0s k = 0.480, P = 0.028). The microbial communities of the samples from Gotland tended to be intermediate between the other Swedish and the German samples, which agrees with the geography of the sampling localities. However, there were no statistically significant differences between locations in Sweden (ANOSIM P = 0.267; Adonis P = 0.343; Wilk0s k = 0.295, P = 0.531). These results were consistent with those obtained from all other distance matrices tested, that is weighted UniFrac, Sorensen, Bray–Curtis and Jaccard (Table S3, Supporting information).

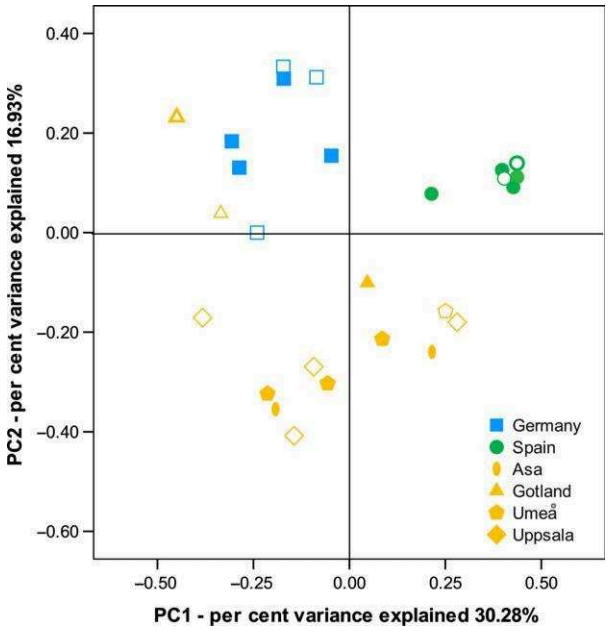

Fig. 3 PCoA plot based on an unweighted UniFrac distance matrix depicting differences in the composition of the gut microbiota from male and female weevils of different locations. Symbols represent community profiles of individual samples. Colors represent countries, shapes represent different locations; full symbols represent females and empty ones males. Thick lines indicate cases in which two communities (male and female) from the same location are identical.

3.4.3. Oligotyping

We used the oligotyping pipeline (Eren *et al.* 2013) to explore bacterial diversity within the four OTUs comprising the 'core microbiota' as well as within the genus *Wolbachia*. The entropy analysis for *Wolbachia* revealed seven positions with high entropy values, all of which were located in homopolymer regions (Fig. S7, Supporting information). As the 454 technology is known to commonly produce errors in homopolymer regions, differences in these regions probably represent sequencing artifacts rather than true biological variation. Thus, we excluded hompolymer substitutions, which left only a single *Wolbachia* oligotype shared by all individuals regardless of their sex and geographical origin.

Entropy analysis of the core microbiota (OTUs 59, 136, 150 and 164) revealed 6, 4, 22 and 6 high entropy nucleotide positions, respectively (Table S5, Supporting information). Following oligotyping and quality filtering, we observed 15, 5, 6 and 10 oligotypes in the members of the core microbiota, respectively. We used a Bray–Curtis dissimilarity matrix to study the differences in oligotype composition between sexes and geographical locations. Statistical analysis showed that there was no difference in the oligotype composition between sexes for any of the core OTUs studied (Table S5, Sup-porting information). Likewise, we found no difference in composition across geographical locations for OTU 136 ($P = 0.1$) and OTU 150 ($P = 0.07$) (Fig. 4). However, the composition significantly differed between locations for OTU 59 ($P = 0.02$) and 164 ($P = 0.01$).

3.4.4. Functional inference

PICRUST predicted metagenome content to level 2 KOs based on the complete data set (i.e. including *Wolbachia*) revealed the putative presence of genes of possible symbiotic importance, such as amino acid, carbohydrate and vitamin metabolism genes (Fig. S8, Supporting information). The PICRUST analysis without *Wolbachia* showed the same gene functions with the addition of xenobiotic degradation and metabolism (Fig. 5). The NSTI values per sample ranged from 0.0089 to 0.03, showing that the 16S rRNA gene of microbes in the weevil0s bacterial metagenome were on average more than 99% similar to those of sequenced genomes in the database.

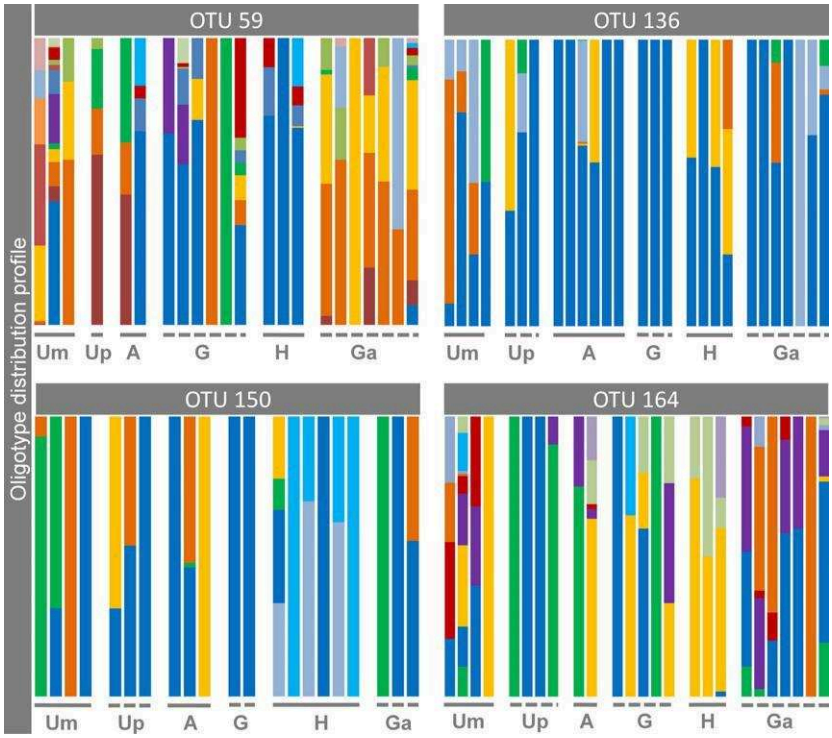

Fig. 4 Oligotype distribution profile of the four 'core' OTUs across geographical locations. Um, Umea; Up, Uppsala; A, Asa; G, Gotland; H, Hannover; Ga, Galicia.

Our independent prediction of the presence of diterpene degradation genes in the pine weevil bacterial metagenome suggested the putative occurrence of a complete *dit* gene cluster. The diterpene degradation cluster contains 20 annotated genes, all of which are likely to be present in the pine weevil's bacterial metagenome, based on the OTUs' closest fully sequenced relatives. Taxonomic classification of these genes showed that most of them were classified as belonging to the genera *Pseudomonas* (which has copies of all genes in the cluster), *Bacillus* (with all copies except dit E, F, G, H and K) and *Sphingomonas* (all genes present except ditN and ditP) (Table S6, Supporting information).

A second homologue search was performed against a CUSTOM database containing fully sequenced genomes of the closest relatives of the pine weevil's core microbiota (i.e. *Rahnella aquatilis, Serratia symbiotica, Serratia odorifera, Yersinia nurmii, Pantoea agglomerans* and *Erwinia typographi*). This

analysis predicts the presence of all genes of the DDGC within these taxa (Table S7, Supporting information).

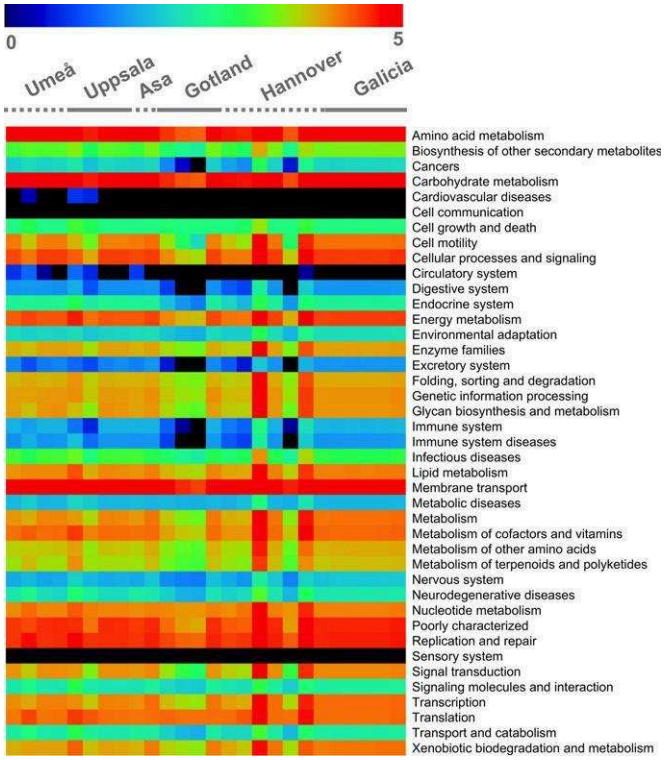

Fig. 5 Heat map in log scale depicting the PICRUSt-inferred gene abundance in the predicted bacterial metagenome of *Hylobius abietis* across different sampling locations in the absence of *Wolbachia*. Warm colors represent high abundances, cold colors represent low abundance and black indicates absence.

3.4.5. Comparison of *Hylobius abietis*' microbial community to those of other conifer-feeding beetles

To study whether *H. abietis* shares a microbiota consistent with that of other conifer-feeding beetles, and to gain some insights as to whether the stability of the pine weevil microbiota may have an ecological significance, we extended our data set with sequences from the gut microbiota of other beetles feeding either on conifers (*Dendroctonus ponderosae, D. frontalis, D. valens*, *D. rhizophagous* and *Ips pini*) or on other food sources (*Rhynchophorus ferrugineus, Otiorhynchus salicicola, O. rugostriatus, O.* sulcatus and *O. armadillo*) (Table S2, Supporting information). We added these sequences to those of *H. abietis* and then reconstructed a phylogenetic tree, which was subsequently simplified by eliminating sequences that did not cluster with those of other beetle-associated bacteria.

Of the 444 sequences that were initially included in the phylogeny, 77 were removed because they clustered distantly from any of the *H. abietis*- or other Curculionidae-associated bacteria. Of the remaining 367 sequences, 123 belonged to bark beetle-associated bacteria and 44 were associated with other beetles, 82 were associated with *H. abietis* and the rest were obtained from the database and

comprised bacteria from many different environments. Of the bark beetle-associated bacterial sequences that were included in the phylogeny, 110 (89.43%) clustered within the Gamma-Proteobacteria, the rest (10.56%) clustered as follows: two within the Alpha-Proteobacteria, four within the Beta-Proteobacteria, two within the Actinobacteria and five within the Firmicutes. Of the 44 bacterial sequences associated with other beetles, only 12 (27.27%) clustered within the Gamma-Proteobacteria, whereas the rest (72.72%) clustered within the Alpha-Proteobacteria (six sequences), Bacteroidetes (four sequences), Beta-Proteobacteria (10 sequences), Actinobacteria (three sequences), Firmicutes (eight sequences) and Chloroflexi (one sequence).

We observed different clustering patterns for the *H. abietis*-associated sequences in Enterobacteriaceae and Firmicutes, while no clustering at all occurred in any other phyla. The Enterobacteriaceae contained the vast majority of *H. abietis*-associated bacterial sequences, which clustered in two main groups (associated with the genera *Erwinia, Rahnella* and *Serratia*) (Fig. 6). These groups were large clusters mainly composed of *H. abietis*- plus bark beetle-associated bacteria, and they contained all four OTUs that constitute *H. abietis*' 'core' microbiota (Fig. 6). By contrast, within the Firmicutes and all other classes, most *H. abietis*-associated sequences occurred dispersed, with the exception of a cluster associated with the genus *Weissella*, which contained no other beetle-associated bacteria (Fig. 7)

Interestingly, some OTUs from *H. abietis* were closely related to those of bark beetles and other weevils, appearing as sister taxa. This occurred in eight cases, in four of which bark beetle associates within the Enterobacteriaceae were the closest relatives, while in four cases, weevil-associated bacteria, specifically from *R. ferrugineus*, were the pine weevil symbionts' sister taxa (twice within the Enterobacteriaceae and twice outside this family). Remarkably, within the Gamma-Proteobacteria, sister taxa occurred with *D. valens, R. ferrugineus* and *D. rhizophagous*, whereas outside that group, it occurred only with *R. ferrugineus*. Thus, while there were some *H. abietis*-associated sequences with high similarity to bacteria from other weevil species feeding on different diets, the most prevalent OTUs clustered with bark beetle-associated microbes in the Enterobacteriaceae.

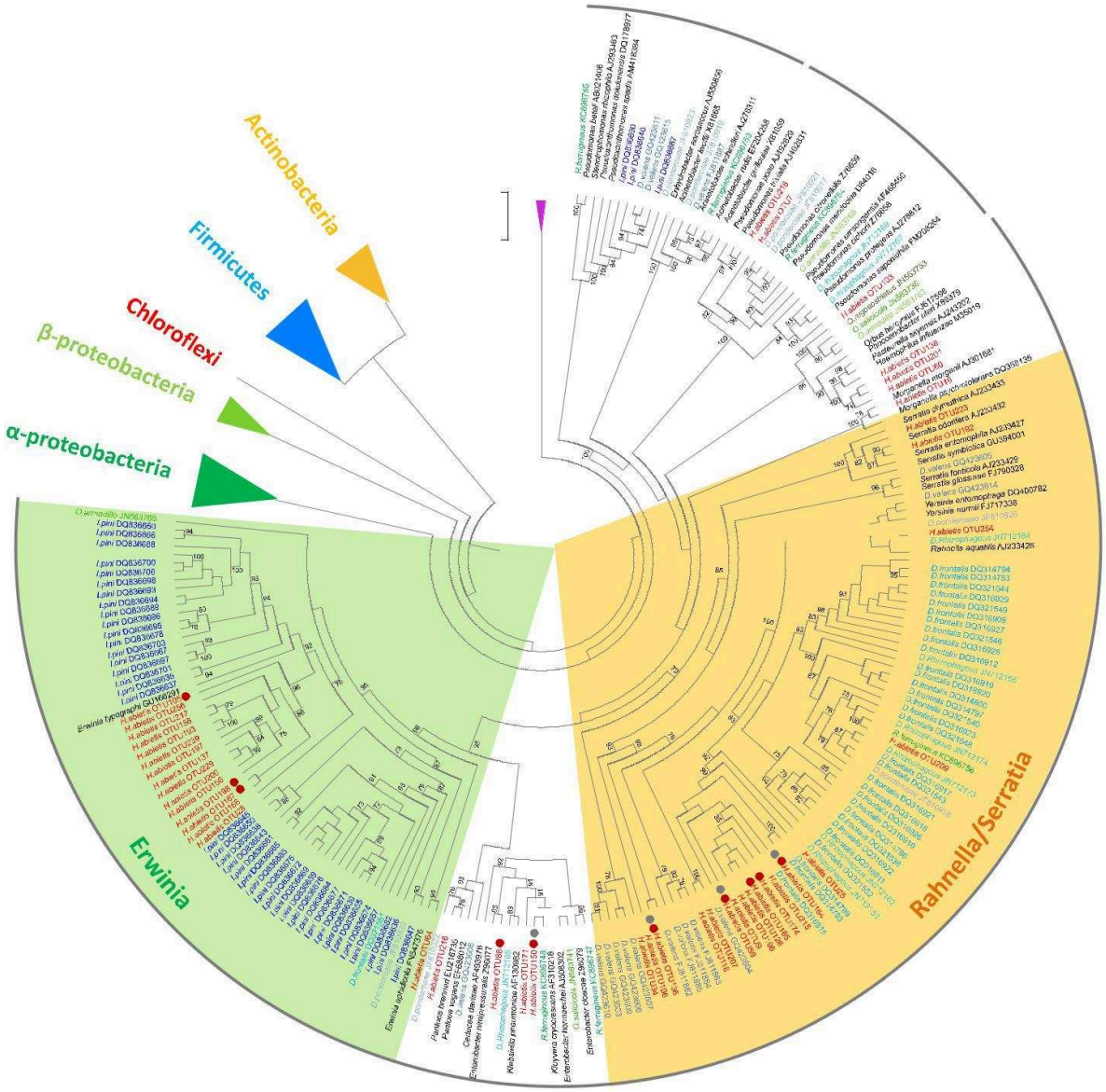

Fig. 6 Phylogenetic placement of the pine weevil gut microbiota (red taxa) in relation to that of other beetles feeding on conifers (blue taxa) or other food sources (green taxa). Only Gamma-Proteobacteria are depicted, all other groups are collapsed (see Fig. 7 for phylogenetic relationships within these groups). Red dots represent OTUs belonging to *Hylobius abietis* that are present in more than 1% abundance in total. Grey dots correspond to OTUs from the 'core microbiota'.

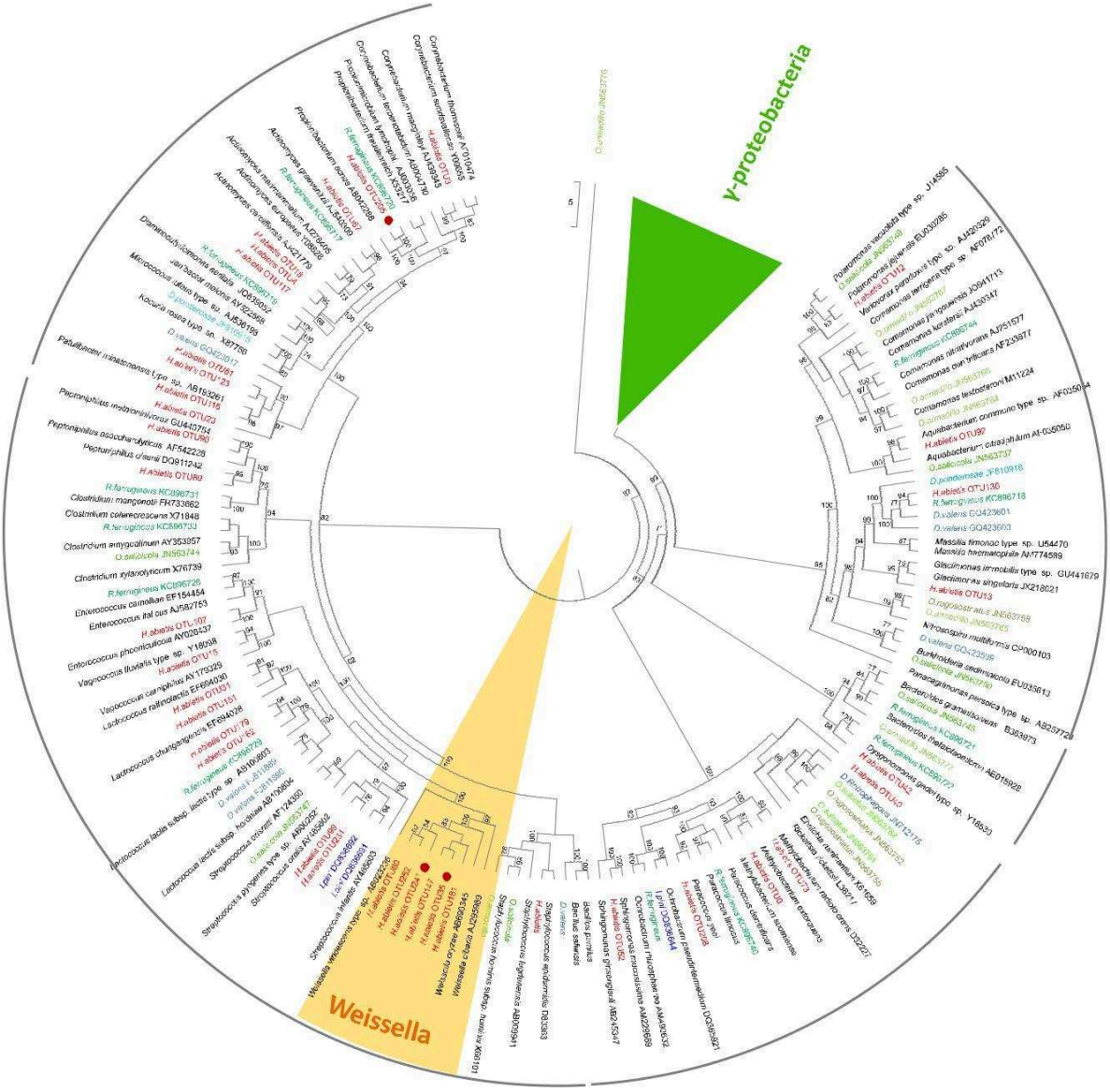

Fig. 7 Phylogenetic placement of the pine weevil gut microbiota (red taxa) in relation to that of other beetles feeding on conifers (blue taxa) or other food sources (green taxa). All bacterial groups except Gamma-Proteobacteria are depicted (see Fig. 6 for phylogenetic relationships within this group). Red dots represent OTUs belonging to *Hylobius abietis* that are present in more than 1% abundance in total.

3.5. DISCUSSION

In the present study, we characterized the gut microbiota of different populations of the European pine weevil, an important pest of young conifer trees in Europe, and compared it to the microbiota of other beetles of the Curculionidae (including other weevils and bark beetles) feeding on the same and different food sources to place the bacterial community in a broader ecological context.

Analyses with both open- and closed-reference OTU picking strategies yielded very similar results. The most striking difference between both methods is the number of OTUs assigned to *Wolbachia*, 28 and 4 for open-reference and closed-reference methods, respectively. However, its abundance remains similar. Taxonomical resolution appears to be better using closed-reference methods. However, this does not affect the results, given that the difference between both approaches within the most abundant taxonomical groups in the pine weevil community is minimal.

We found *Wolbachia* to be present in all samples studied. The analysis of the microbiota with QIIME showed 28 OTUs binned to *Wolbachia*. However, oligotyping analysis revealed that all pine weevils harboured just one oligotype (a unique 16S rRNA sequence), indicating that all individuals share just one strain of *Wolbachia* regardless of sex or geographical location. This discordance on the number of *Wolbachia* strains is most likely due to the fact that the highly variable nucleotides found in the 16S rRNA sequence were located in homopolymer regions and thus are probably the result of sequencing errors (Quince *et al.* 2011). *Wolbachia* infects up to two-thirds of all known insects (Hilgenboecker *et al.* 2008), and it is most well-known for its manipulative effects on host reproduction (Werren *et al.* 2008), but mutualistic associations for nutrition (Hosokawa *et al.* 2010) or defense (Hedges *et al.* 2008) of the host have also been described. In a previous study, 40% of sampled weevil species from central Europe were infected with *Wolbachia* (Lachowska *et al.* 2010). *Wolbachia* is most commonly found in insect reproductive tissues, but localization in other tissues including the gut has also been described (Dobson *et al.* 1999; Frost *et al.* 2014). In *H. abietis*, as has been speculated for *Acromyrmex* leaf-cutting ants (Andersen *et al.* 2012), the presence of *Wolbachia* in the gut may point towards a nutritional function, although reproductive manipulation or a commensal relationship cannot be ruled out without further experiments.

After removing *Wolbachia* sequences from the analysis, Proteobacteria and Firmicutes turned out to be the most abundant and prevalent phyla in the pine weevil microbiota, followed by Actinobacteria. Even though the composition of insect-associated microbial communities differs strongly among insect species, Proteobacteria and Firmicutes appear to be the most prevalent phyla (Colman *et al.* 2012). Both groups have also been described to be dominant in the conifer-feeding bark beetles, *Dendroctonus valens* and *I. pini* (Delalibera *et al.* 2007; Morales-Jimenez *et al.* 2009). More specifically, we found Enterobacteriaceae to be the most dominant family harbored in the pine weevil gut. This family has also been reported in bark beetles such as *D. ponderosae* (Adams *et al.* 2013), *D. frontalis* (Vasanthakumar *et al.* 2006), *Dendroctonus rhizopagus* (Morales-Jimenez *et al.* 2012) and in *I. pini* (Delalibera *et al.* 2007).

While bacterial communities of insect guts can exhibit large variation in space and time (Toju and Fukatsu 2011; Zouache *et al.* 2011), a number of studies have nonetheless demonstrated that functionally relevant microbial communities can be remarkably stable, as exemplified in firebugs (Sudakaran *et al.* 2012), honey bees (Martinson *et al.* 2011) and termites (Hongoh *et al.* 2005). In bark beetles, the geographical stability of microbial communities varies across host taxa, being high in *D. ponderosae* (Adams *et al.* 2013) and low in *D. valens* (Adams *et al.* 2010), although some microbial taxa were always present in the latter regardless of sampling location. Our data demonstrate a rather stable gut community in the pine weevil across different locations in Europe (Fig. 1). Around 50% of the most prevalent OTUs were present in every country, and members of the Enterobacteriaceae were present in every location. In

particular, two oligotypes of this family were geographically stable and were present in all locations studied. However, differences between geographical locations were also detected (both at OTU and oligotype levels), resulting in the significant separation of sampling locations based on the beetles' microbial community profiles (Fig. 3). Interestingly, individuals that presented a high abundance of Proteobacteria harbored a low abundance of Firmicutes and vice versa (Fig. 2), which could suggest mutual competitive exclusion of these two groups in the pine weevil's gut.

Colman and colleagues (2012) found that beetles feeding on bark and phloem of living trees harbored similar bacterial taxa and that their microbial communities generally exhibited low richness and phylogenetic diversity. We found the overall bacterial richness in guts of adult pine weevils (16 8 OTUs/sample) to be slightly higher than the richness in guts of bark beetles (e.g. 5 and 3 OTUs/sample), based on Chao1 richness estimators after OTU clustering with 97% similarity thresholds (Vasanthakumar *et al.* 2006; Morales-Jimenez *et al.* 2009). Several factors may explain the higher diversity detected in our study as compared to previous reports on bark beetles. First, we screened a greater number of samples in our analysis compared to previous studies, which may have contributed to an increased OTU number. Second, we used 454 pyrosequencing as opposed to DGGE, which—due to its greater depth and sensitivity, but also the potential generation of sequencing artifacts—is known to yield more diverse community profiles (Gilles *et al.* 2011; Quince *et al.* 2011). Despite the higher microbial diversity in our study, we could confirm that conifer phloem-feeding beetles harbor species-poor communities compared to those of other insects, including xylophagous taxa (e.g. 89 61 OTUs/sample in Isoptera) and detritivorous taxa (53.2 33 OTUs/sample) (Colman *et al.* 2012). The low bacterial richness in conifer phloem-feeding beetles may reflect the antimicrobial properties of the toxic defensive chemicals encountered in conifer phloem, such as terpenoids (Trombetta *et al.* 2005; Adams *et al.* 2011). Bacteria from the Enterobacteriaceae family are known for their frequent association with insects as intra- as well as extracellular nutritional symbionts (Baumann 2005; Lauzon *et al.* 2009; Husnik *et al.* 2011; Nikoh *et al.* 2011). The recurrent presence of Enterobacteriaceae taxa in the pine weevil whose closest relatives are *Rahnella, Serratia, Pantoea* and *Erwinia*, the phylogenetic relatedness of these microbes to those harbored by different species of bark beetles and our functional prediction of the bacterial metagenome suggest that they are common associates of conifer phloem-feeding insects and might play an important role in overcoming the nutritional challenges that this diet poses. Specifically, the low concentration of nitrogen could be overcome by the association with nitrogen-fixing bacteria. *Enterobacter* spp. are known to fix atmospheric nitrogen in the apple maggot (Lauzon *et al.* 2000) as well as in termites (Potrikus and Breznak 1977), and they have been isolated from *Dendroctonus terebrans* (Bridges 1981). Additionally, *Pantoea, Rahnella aquatilis* and *Serratia* spp. are known to fix nitrogen in plant–microbe associations (Behar *et al.* 2005) and could potentially perform the same function in an insect host. Furthermore, *Serratia* spp., *Erwinia* sp. and *Enterobacter aerogenes* are facultatively anaerobic bacteria that have been proposed to generate micro-anaerobic sites in termite guts where nitrogen fixation as well as cellulose degradation can take place (Adams and Boopathy 2005).

Some members of the pine weevil microbial community could also be involved in the degradation of plant chemical defenses. *Pseudomonas* spp. and close relatives of *Rahnella* spp. are known for their ability to degrade aromatic compounds (Sarma *et al.* 2004; Wongsa *et al.* 2004; Bicas *et al.* 2008). Moreover, the bac-terial metagenome of *D. ponderosae* (Adams *et al.* 2013) contains a complete gene cluster involved in diterpene degradation. Adams *et al.* (2013) were able to match some of those genes to specific members of the bacterial community, that is Pseudomonas, *Rahnella, Stenotrophomonas, Serratia, Pantoea, Erwinia*, as well as *Burkholderia*, most of which are found in *H. abietis*' gut community in high abundances. Isolates from some of those taxa can degrade terpenes in vitro (Boone *et al.* 2013) and do so more efficiently at concentrations typical of constitutive rather than induced chemical defenses (Raffa 2014). Our functional inference of the bacterial metagenome suggests as much, indicating that *Pseudomonas* sp., *Bacillus* sp. and *Sphingomonas* sp, as well as members of the genera *Rahnella, Serratia,*

Erwinia, Pantoea among others, are likely to contain a *dit* gene cluster and hence could be involved in such detoxification. Interestingly, it seems that although there is some degree of metabolic redundancy, *D. ponderosae's* gut community appears to be highly complementary from a metabolic standpoint, as distinct bacterial species have been found to degrade different terpenoids in vitro (Boone *et al.* 2013). Thus, given (i) the overlap in the microbial community between bark beetles and the pine weevil, (ii) the presence of a complete DDGC in the bacterial metagenome of the bark beetle *D. ponderosae*, (iii) the ability of some microbes that are closely related to *H. abietis*-associated bacteria to degrade diterpenes in vitro and (iv) the putative presence of diterpene degradation genes in the pine weevil's bacterial metagenome, it seems likely that the same taxa could be involved in the detoxification of terpenes in the pine weevil.

Besides diet, phylogenetic relatedness is one of the main factors shaping insect microbial communities (Colman *et al.* 2012; Ravussin *et al.* 2012). Indeed, all beetles compared in this study belong to the Curculionidae family and it is plausible that the shared microbiota found in these insects reflects their relatedness. However, outside this beetle family, a similar bacterial community has been described in a species of the Cerambycidae (*Monochamus galloprovincialis*) sampled in Portugal (Vicente *et al.* 2013), which also lives on conifers (i.e. *Pinus pinaster*). The similarity of the pine weevil's microbiota to that of a cerambycid beetle and to bark beetles suggests that the ecological niche plays an important role in shaping the community of these insects. Distantly related insects exploiting similar ecological niches have previously been shown to harbor similar microbiotas or at least equivalent functional diversity (Muegge *et al.* 2011; Fan *et al.* 2012).

The taxonomic convergence of the microbiota of conifer-feeding beetles is remarkable given that all these insects have been sampled from distant locations spanning two continents. However, the functional homology of these bacterial taxa among different species of beetles remains to be confirmed by further metagenomic, transcriptomic and biochemical analyses. The similarity in the gut microbiota of different conifer-feeding beetles could be explained by two different hypotheses. The most likely scenario is that many of the microbial taxa found in this study are frequent residents of conifer tissue due to their ability to degrade specific conifer metabolites. Hence, they may be horizontally acquired by the insect and present only transiently in the gut. Alternatively, vertical transmission from mother to offspring may occur to ensure that a functionally relevant microbiota is passed from generation to generation. However, occasional horizontal acquisition of gut microbes would still be necessary to explain the geographical patterns observed for *H. abietis* in this study. Under both scenarios, the observed presence of conserved bacterial taxa in different species of conifer-feeding beetles from distant locations, and the putative occurrence of genes involved in terpenoid degradation, suggests that these microbes are of functional importance to their hosts.

Wood boring beetles such as the pine weevil and bark beetles represent a serious threat to conifer forests world-wide. In Europe, *H. abietis* and *Ips typography* are the most damaging conifer pests with a reported distribution spanning more than a dozen countries (Gregoire and Evans 2004, Langstrom and Day 2004). The pine weevil alone has caused very serious damage in more than 88 258 ha between 1980 and 2000, and 3 418 264 ha is considered to be under threat (Gregoire and Evans 2004). In North America, bark beetles also represent a serious ecological threat. Understanding the interaction between these insects, their symbionts and the conifer hosts at different levels (i.e. ecological, physiological and molecular) is key to understanding their ability to devastate forests and can potentially help in designing more efficient strategies to protect these ecosystems.

3.6. ACKNOWLEDGEMENTS

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3.7. REFERENCES

Adams L, Boopathy R (2005) Isolation and characterization of enteric bacteria from the hindgut of Formosan termite. *Bioresource Technology*, 96, 1592–1598.

Adams AS, Adams SM, Currie CR, Gillette NE, Raffa KF (2010) Geographic variation in bacterial communities associated with the red turpentine beetle (Coleoptera: Curculionidae). *Environmental Entomology*, 39, 406–414.

Adams AS, Boone CK, Bohlmann J, Raffa KF (2011) Responses of bark beetle-associated bacteria to host monoterpenes and their relationship to insect life histories. *Journal of Chemical Ecology*, 37, 808–817.

Adams AS, Aylward FO, Adams SM *et al.* (2013) Mountain pine beetles colonizing historical and naive host trees are associated with a bacterial community highly enriched in genes contributing to terpene metabolism. *Applied and Environmental Microbiology*, 79, 3468–3475.

Andersen SB, Boye M, Nash DR, Boomsma JJ (2012) Dynamic *Wolbachia* prevalence in Acromyrmex leaf-cutting ants: potential for a nutritional symbiosis. *Journal of Evolutionary Biology*, 25, 1340–1350.

Ayres MP, Wilkens RT, Ruel JJ, Lombardero MJ, Vallery E (2000) Nitrogen budgets of phloem-feeding bark beetles with and without symbiotic fungi. *Ecology*, 81, 2198–2210.

Barras SJ (1967) Thoracic mycangium of *Dendroctonus frontalis* (Coleoptera – Scolytidae) is synonymous with a secondary female character. *Annals of the Entomological Society of America,* 60, 486–487.

Baumann P (2005) Biology of bacteriocyte-associated endosymbionts of plant sap-sucking insects. *Annual Review of Microbiology*, 59, 155–189.

Behar A, Yuval B, Jurkevitch E (2005) Enterobacteria-mediated nitrogen fixation in natural populations of the fruit fly *Ceratitis capitata. Molecular Ecology*, 14, 2637–2643.

Bentz BJ, Six DL (2006) Ergosterol content of fungi associated with *Dendroctonus ponderosae* and *Dendroctonus rufipennis* (Coleoptera: Curculionidae, Scolytinae). *Annals of the Entomological Society of America*, 99, 189–194.

Berryman AA (1976) Theoretical explanation of mountain pine beetle dynamics in lodgepole pine forests. *Environmental Entomology*, 5, 1225–1233.

Bicas JL, Fontanille P, Pastore GM, Larroche C (2008) Characterization of monoterpene biotransformation in two pseudomonads. *Journal of Applied Microbiology*, 105, 1991– 2001.

Bleiker KP, Six DL (2007) Dietary benefits of fungal associates to an eruptive herbivore: potential implications of multiple associates on host population dynamics. *Environmental Entomology*, 36, 1384–1396.

Boone CK, Keefover-Ring K, Mapes AC *et al.* (2013) Bacteria associated with a tree-killing insect reduce concentrations of plant defense compounds. *Journal of Chemical Ecology*, 39, 1003–1006.

Bridges JR (1981) Nitrogen-fixing bacteria associated with bark beetles. *Microbial Ecology*, 7, 131–137.

Caporaso JG, Kuczynski J, Stombaugh J *et al.* (2010) QIIME allows analysis of high-throughput community sequencing data. *Nature Methods*, 7, 335–336.

Codella SG, Raffa KF (1995) Contributions of female oviposition patterns and larval behavior to group defense in conifer sawflies (Hymenoptera, Diprionidae). *Oecologia*, 103, 24–33.

Colman DR, Toolson EC, Takacs-Vesbach CD (2012) Do diet and taxonomy influence insect gut bacterial communities? *Molecular Ecology*, 21, 5124–5137.

Conesa A, Götz S, Garcia-Gomez JM, Terol J, Talon M, Robles M (2005) Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics research. *Bioinformatics*, 21, 3674–3676.

Craighead FC, George RAS (1940) Field observations on the dying of pines infected with the blue-stain fungus, *Ceratostomella pini* Munch. *Phytopathology*, 30, 976–979.

Delalibera I, Vasanthakumar A, Burwitz BJ *et al.* (2007) Composition of the bacterial community in the gut of the pine engraver, *Ips pini* (Say) (Coleoptera) colonizing red pine. *Symbiosis*, 43, 97–104.

Deneubourg JL, Gregoire JC, Lefort E (1990) Kinetics of larval gregarious behavior in the bark beetle *Dendroctonus micans* (Coleoptera, Scolytidae). *Journal of Insect Behavior*, 3, 169– 182.

DiGuistini S, Wang Y, Liao NY *et al.* (2011) Genome and transcriptome analyses of the mountain pine beetle-fungal symbiont *Grosmannia clavigera*, a lodgepole pine pathogen. *Proceedings of the National Academy of Sciences of the United States of America*, 108, 2504–2509.

Dobson SL, Bourtzis K, Braig HR *et al.* (1999) *Wolbachia* infections are distributed throughout insect somatic and germ line tissues. *Insect Biochemistry and Molecular Biology*, 29, 153–160.

Douglas AE (2009) The microbial dimension in insect nutritional ecology. *Functional Ecology*, 23, 38–47.

Eren AM, Maignien L, Sul WJ *et al.* (2013) Oligotyping: differentiating between closely related microbial taxa using 16S rRNA gene data. *Methods in Ecology and Evolution*, 4, 1111– 1119.

Fan L, Reynolds D, Liu M *et al.* (2012) Functional equivalence and evolutionary convergence in complex communities of microbial sponge symbionts. *Proceedings of the National Academy of Sciences of the United States of America*, 109, E1878– E1887.

Feltrer R, Alvarez-Rodriguez ML, Barreiro C, Godio RP, Coque JJR (2010) Characterization of a novel 2,4,6-trichlorophenol-inducible gene encoding chlorophenol O-methyltransferase from *Trichoderma longibrachiatum* responsible for the formation of chloroanisoles and detoxification of chlorophenols. *Fungal Genetics and Biology*, 47, 458–467.

Frost CL, Pollock SW, Smith JE, Hughes WOH (2014) *Wolbachia* in the flesh: symbiont intensities in germline and somatic tissues challenge the conventional view of *Wolbachia* transmission routes. *PLoS One*, 9, e95122.

Gilles A, Meglecz E, Pech N *et al.* (2011) Accuracy and quality assessment of 454 GS-FLX Titanium pyrosequencing. *BMC Genomics*, 12, 245.

Gregoire JC, Braekman JC, Tondeur A (1981) Chemical communication between the larvae of *Dendroctonus micans* Kug (Coleoptera: Scolytidae). Les colloques de L'INRA, 7 Les mediateurs chimiques, 253–257.

Gre´goire JC, Evans HF (2004) Damage and control of BAW-BILT organisms, an overview. In: Bark and Wood Boring Insects in Living Trees in Europe, a Synthesis. (eds Lieutier F, Day KR, Battisti A, Gregoire JC, Evans HF), pp. 19–37. Kluwer, Dordrecht.

Grossmann H (1930) Beiträge zur kenntnis der lebensgemeinschaft zwischen borkenkäfern und pilzen. Zeitschrift für Parasitenkunde, 3, 56–102.

Hedges LM, Brownlie JC, O'Neill SL, Johnson KN (2008) *Wolbachia* and virus protection in insects. *Science*, 322, 702.

Hilgenboecker K, Hammerstein P, Schlattmann P, Telschow A, Werren JH (2008) How many species are infected with *Wolbachia*? – a statistical analysis of current data. *FEMS Microbiology Letters*, 281, 215–220.

Hongoh Y, Deevong P, Inoue T *et al.* (2005) Intra- and inter-specific comparisons of bacterial diversity and community structure support coevolution of gut microbiota and termite host. *Applied and Environmental Microbiology*, 71, 6590–6599.

Hosokawa T, Koga R, Kikuchi Y, Meng XY, Fukatsu T (2010) *Wolbachia* as a bacteriocyte-associated nutritional mutualist. *Proceedings of the National Academy of Sciences of the USA*, 107, 769–774.

Husnik F, Chrudimsky T, Hypsa V (2011) Multiple origins of endosymbiosis within the Enterobacteriaceae (gamma-Proteobacteria): convergence of complex phylogenetic approaches. *BMC Biology*, 9, 87.

Kanehisa M, Goto S, Sato Y, Furumichi M, Tanabe M (2012) KEGG for integration and interpretation of large-scale molecular data sets. *Nucleic Acids Research*, 40, D109–D114.

Keeling CI, Bohlmann J (2006) Diterpene resin acids in conifers. *Phytochemistry*, 67, 2415–2423.

Lachowska D, Kajtoch L, Knutelski S (2010) Occurrence of *Wolbachia* in central European weevils: correlations with host systematics, ecology, and biology. *Entomologia Experimentalis et Applicata*, 135, 105–118.

Langille MGI, Zaneveld J, Caporaso JG *et al.* (2013) Predictive functional profiling of microbial communities using 16S rRNA marker gene sequences. *Nature Biotechnology*, 31, 814–821.

Langstrom B, Day KR (2004) Damage control and management of weevil pests, especially *Hylobius abietis*. In: Bark and Wood Boring Insects in Living Trees in Europe, a Synthesis. (eds Lieutier F, Day KR, Battisti A, Gregoire JC, Evans HF), pp. 415–444. Kluwer, Dordrecht.

Lauzon CR, Sjogren RE, Prokopy RJ (2000) Enzymatic capabilities of bacteria associated with apple maggot flies: a postulated role in attraction. *Journal of Chemical Ecology*, 26, 953–967.

Lauzon CR, McCombs SD, Potter SE, Peabody NC (2009) Establishment and vertical passage of *Enterobacter* (*Pantoea) agglomerans* and *Klebsiella pneumoniae* through all life stages of the Mediterranean fruit fly (Diptera: Tephritidae). *Annals of the Entomological Society of America*, 102, 85–95.

Leather SR, Day KR, Salisbury AN (1999) The biology and ecology of the large pine weevil, *Hylobius abietis* (Coleoptera: Curculionidae): a problem of dispersal? *Bulletin of Entomological Research*, 89, 3–16.

Li SH, Nagy NE, Hammerbacher A *et al.* (2012) Localization of phenolics in phloem parenchyma cells of Norway spruce (*Picea abies*). *ChemBioChem*, 13, 2707–2713.

Lindh JM, Terenius O, Faye I (2005) 16S rRNA gene-based identification of midgut bacteria from field-caught *Anopheles gambiae sensu lato* and *A. funestus* mosquitoes reveals new species related to known insect symbionts. *Applied and Environmental Microbiology*, 71, 7217–7223.

Ludwig W, Strunk O, Westram R *et al.* (2004) ARB: a software environment for sequence data. *Nucleic Acids Research*, 32, 1363–1371.

Martinson VG, Danforth BN, Minckley RL *et al.* (2011) A simple and distinctive microbiota associated with honey bees and bumble bees. *Molecular Ecology*, 20, 619–628.

McCullough DC, Wagner MR (1993) Defusing Host Defenses: Ovipositional Adaptations of Sawflies to Plant Resins. Academic Press, San Diego.

Morales-Jimenez J, Zuniga G, Villa-Tanaca L, Hernandez-Rodriguez C (2009) Bacterial community and nitrogen fixation in the red turpentine beetle, *Dendroctonus valens* LeConte (Coleoptera: Curculionidae: Scolytinae). *Microbial Ecology*, 58, 879–891.

Morales-Jimenez J, Zuniga G, Ramirez-Saad HC, Hernandez-Rodriguez C (2012) Gut-associated bacteria throughout the life cycle of the bark beetle *Dendroctonus rhizophagus* Thomas and Bright (Curculionidae: Scolytinae) and their cellulolytic activities. *Microbial Ecology*, 64, 268–278.

Muegge BD, Kuczynski J, Knights D *et al.* (2011) Diet drives convergence in gut microbiome functions across mammalian phylogeny and within humans. *Science*, 332, 970–974.

Nikoh N, Hosokawa T, Oshima K, Hattori M, Fukatsu T (2011) Reductive evolution of bacterial genome in insect gut environment. *Genome Biology and Evolution*, 3, 702–714.

Nordlander G (1987) A method for trapping *Hylobius abietis* (L.) with a standardized bait and its potential for forecasting seedling damage. *Scandinavian Journal of Forest Research*, 2, 199–213.

Nordlander G, Nordenhem H, Bylund H (1997) Oviposition patterns of the pine weevil *Hylobius abietis*. *Entomologia Experimentalis et Applicata*, 85, 1–9.

Nordlander G, Bylund H, Bjorklund N (2005) Soil type and microtopography influencing feeding above and below ground by the pine weevil *Hylobius abietis*. *Agricultural and Forest Entomology*, 7, 107–113.

Nordlander G, Hellqvist C, Johansson K, Nordenhem H (2011) Regeneration of European boreal forests: effectiveness of measures against seedling mortality caused by the pine weevil *Hylobius abietis*. *Forest Ecology and Management*, 262, 2354–2363.

Oliveros JC (2007) VENNY. An interactive tool for comparing lists with Venn Diagrams. Available at: http://bioin-fogp.cnb.csic.es/tools/venny/index.html.

Paine TD, Raffa KF, Harrington TC (1997) Interactions among scolytid bark beetles, their associated fungi, and live host conifers. *Annual Review of Entomology*, 42, 179–206.

Petersson M, Orlander G (2003) Effectiveness of combinations of shelterwood, scarification, and feeding barriers to reduce pine weevil damage. *Canadian Journal of Forest Research* – Revue Canadienne De Recherche Forestiere, 33, 64–73.

Pignal MC, Chararas C, Bourgeaycausse M (1988) Yeasts from *Ips sexdentatus* (Scolytidae) – enzymatic activity and vitamin excretion. *Mycopathologia*, 103, 43–48.

Potrikus CJ, Breznak JA (1977) Nitrogen-fixing *Enterobacter agglomerans* isolated from guts of wood-eating termites. *Applied and Environmental Microbiology*, 33, 392–399.

Price MN, Dehal PS, Arkin AP (2009) FastTree: computing large minimum-evolution trees with profiles instead of a distance matrix. *Molecular Biology and Evolution*, 26, 1641–1650.

Price MN, Dehal PS, Arkin AP (2010) FastTree 2: approximately Maximum-Likelihood trees for large alignments. *PLoS One*, 5, e9490.

Pruesse E, Peplies J, Glockner FO (2012) SINA: accurate high-throughput multiple sequence alignment of ribosomal RNA genes. *Bioinformatics*, 28, 1823–1829.

Quince C, Lanzen A, Davenport RJ, Turnbaugh PJ (2011) Removing noise from pyrosequenced amplicons. *BMC Bioinformatics*, 12, 38.

Raffa KF (2014) Terpenes tell different tales at different scales: glimpses into the chemical ecology of conifer – bark beetle – microbial interactions. *Journal of Chemical Ecology*, 40, 1–20.

Ravussin Y, Koren O, Spor A *et al.* (2012) Responses of gut microbiota to diet composition and weight loss in lean and obese mice. *Obesity*, 20, 738–747.

Saeed AI, Sharov V, White J *et al.* (2003) TM4: a free, open-source system for microarray data management and analysis. *BioTechniques*, 34, 374–378.

Sarma PM, Bhattacharya D, Krishnan S, Lal BW (2004) Degradation of polycyclic aromatic hydrocarbons by a newly discovered enteric bacterium, *Leclercia adecarboxylata*. *Applied and Environmental Microbiology*, 70, 3163–3166.

Six DL, Paine TD (1998) Effects of mycangial fungi and host tree species on progeny survival and emergence of *Dendroctonus ponderosae* (Coleoptera: Scolytidae). *Environmental Entomology*, 27, 1393–1401.

Smith DJ, Martin VJJ, Mohn WW (2004) A cytochrome P450 involved in the metabolism of abietane diterpenoids by *Pseudomonas abietaniphila* BKME-9. *Journal of Bacteriology*, 186, 3631–3639.

Solbreck C (1980) Dispersal distances of migrating pine weevils, *Hylobius abietis*, Coleoptera – Curculionidae. *Entomologia Experimentalis et Applicata*, 28, 123–131.

Solbreck C, Gyldberg B (1979) Temporal flight pattern of the large pine weevil, *Hylobius abietis* L (Coleoptera, Curculionidae), with special reference to the influence of weather. *Zeitschrift Fur Angewandte Entomologie – Journal of Applied Entomology*, 88, 532–536.

Strongman DB (1987) A method for rearing *Dendroctonus ponderosae* Hopk (Coleoptera, Scolytidae) from eggs to pupae on host tissue with or without a fungal complement. *The Canadian Entomologist*, 119, 207–208.

Sudakaran S, Salem H, Kost C, Kaltenpoth M (2012) Geographical and ecological stability of the symbiotic mid-gut micro-biota in European firebugs, *Pyrrhocoris apterus* (Hemiptera, Pyrrhocoridae). *Molecular Ecology*, 21, 6134–6151.

Swanson KS, Dowd SE, Suchodolski JS *et al.* (2011) Phylogenetic and gene-centric metagenomics of the canine intestinal microbiome reveals similarities with humans and mice. *ISME Journal*, 5, 639–649.

Tamura K, Peterson D, Peterson N *et al.* (2011) MEGA5: Molecular Evolutionary Genetics Analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular Biology and Evolution*, 28, 2731–2739.

Thornber JP, Northcote DH (1961a) Changes in the chemical composition of a cambial cell during its differentiation into xylem and phloem tissue in trees. I. Main components. *Biochemical Journal*, 81, 449–455.

Thornber JP, Northcote DH (1961b) Changes in the chemical composition of a cambial cell during its differentiation into xylem and phloem tissue in trees. II. Carbohydrate constituents of each main component. *Biochemical Journal*, 81, 455–464.

Thornber JP, Northcote DH (1962) Changes in the chemical composition of a cambial cell during its differentiation into xylem and phloem tissue in trees. III. Xylan, glucomannan and alpha-cellulose fractions. *Biochemical Journal*, 82, 340– 346.

Toju H, Fukatsu T (2011) Diversity and infection prevalence of endosymbionts in natural populations of the chestnut weevil: relevance of local climate and host plants. *Molecular Ecology*, 20, 853–868.

Trombetta D, Castelli F, Sarpietro MG *et al.* (2005) Mechanisms of antibacterial action of three monoterpenes. *Antimicrobial Agents and Chemother*apy, 49, 2474–2478.

Vasanthakumar A, Delalibera I, Handelsman J *et al.* (2006) Characterization of gut-associated bacteria in larvae and adults of the southern pine beetle, *Dendroctonus frontalis* Zimmermann. *Environmental Entomology*, 35, 1710–1717.

Vicente CSL, Nascimento FX, Espada M *et al.* (2013) Characterization of bacterial communities associated with the pine sawyer beetle *Monochamus galloprovincialis*, the insect vector of the pinewood nematode *Bursaphelenchus xylophilus*. *FEMS Microbiology Letters*, 347, 130–139.

Wainhouse D, Inward DJG, Morgan G (2014) Modelling geographical variation in voltinism of *Hylobius abietis* under climate change and implications for management. *Agricultural and Forest Entomology*, 16, 136–146.

Wallertz K, Nordlander G, Orlander G (2006) Feeding on roots in the humus layer by adult pine weevil, *Hylobius abietis*. *Agricultural and Forest Entomology*, 8, 273–279.

Wang Q, Garrity GM, Tiedje JM, Cole JC (2007) Naive bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Applied and Environmental Microbiology*, 73, 5261–5267.

Wang Y, Lim L, DiGuistini S *et al.* (2013) A specialized ABC efflux transporter GcABC-G1 confers monoterpene resistance to *Grosmannia clavigera*, a bark beetle-associated fungal pathogen of pine trees. *New Phytologist*, 197, 886–898.

Warren CR, Adams MA (2002) Phosphorus affects growth and partitioning of nitrogen to Rubisco in *Pinus pinaster*. *Tree Physiology*, 22, 11–19.

Werren JH, Baldo L, Clark ME (2008) *Wolbachia*: master manipulators of invertebrate biology. *Nature Reviews Microbiology*, 6, 741–751.

Whitney HS, Cobb FW (1972) Non-staining fungi associated with bark beetle *Dendroctonus brevicomis* (Coleoptera-Scolytidae) on *Pinus ponderosa*. *Canadian Journal of Botany*, 50, 1943–1945.

Wöll S, Kim SH, Greten HJ, Efferth T (2013) Animal plant warfare and secondary metabolite evolution. *Natural Products and Bioprospecting*, 3, 1–7.

Wongsa P, Tanaka M, Ueno A *et al.* (2004) Isolation and characterization of novel strains of *Pseudomonas aeruginosa* and *Serratia marcescens* possessing high efficiency to degrade gasoline, kerosene, diesel oil, and lubricating oil. *Current Microbiology*, 49, 415–422.

Zouache K, Raharimalala FN, Raquin V *et al.* (2011) Bacterial diversity of field-caught mosquitoes, *Aedes albopictus* and *Aedes aegypti*, from different geographic regions of Madagascar. *FEMS Microbiology Ecology*, 75, 377–389.

3.8. AUTHOR CONTRIBUTIONS

A.B., M.K., A.S., J.G., A.K.B.K. and O.T. conceived the study; A.B. and K.A. collected weevils, K.A. per-formed dissections, O.T. performed DNA extraction and 454 sequencing preparation, and A.B. performed the analyses. A.B. and M.K. wrote the manuscript, which was edited and agreed for publication by all authors.

3.9. DATA ACCESSIBILITY

Raw fasta files with complete set of bacterial 16S rRNA sequences, quality filtered fasta files, alignment files, phylogenetic tree files and mapping files are available on Dryad (doi: 10.5061/dryad.f3r67.2).

3.10. SUPPORTING INFORMATION

Figure S1. Map of Europe depicting the different sampling locations of *H. abietis*.

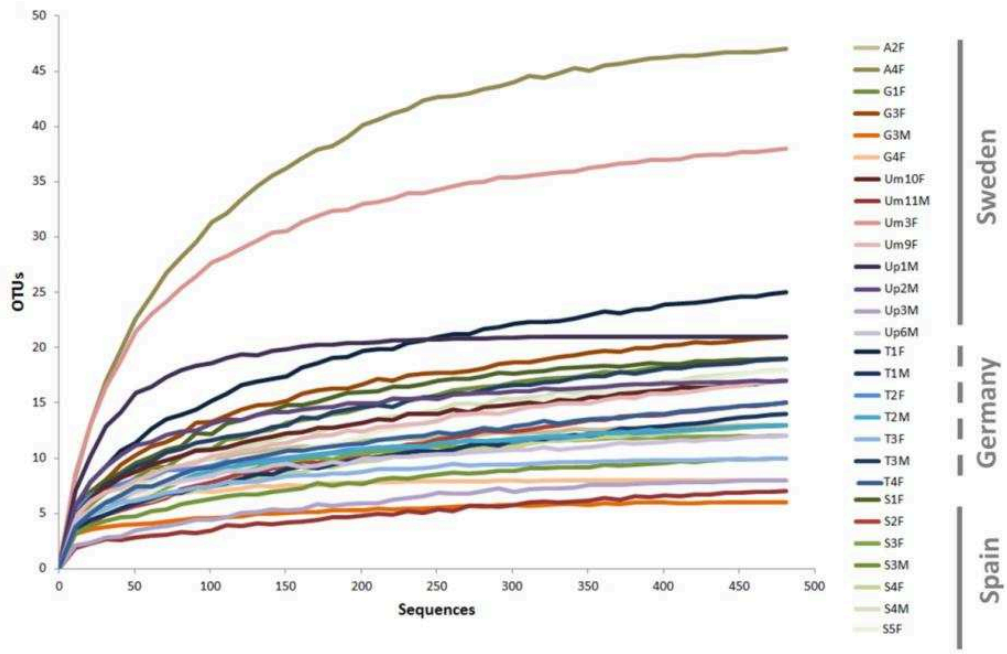

Figure S2. Rarefaction curves of bacterial 16S rRNA gene sequences from weevil gut samples from different geographical locations after eliminating Wolbachia sequences. Sequences were binned into OTUs at a 97% sequence similarity cut-off value. A, Asa; G, Gotland; Um, Umeå; Up, Uppsala.

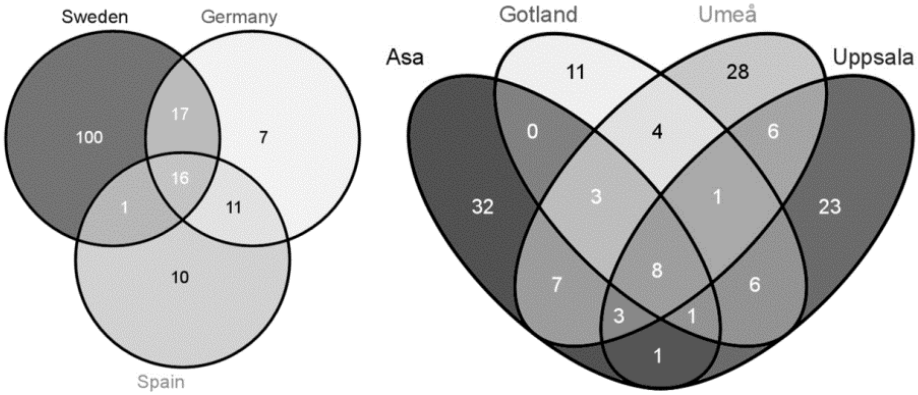

Figure S3. Venn diagram depicting the number of shared and exclusive bacterial OTUs in *H. abietis'* gut across countries and across locations in Sweden.

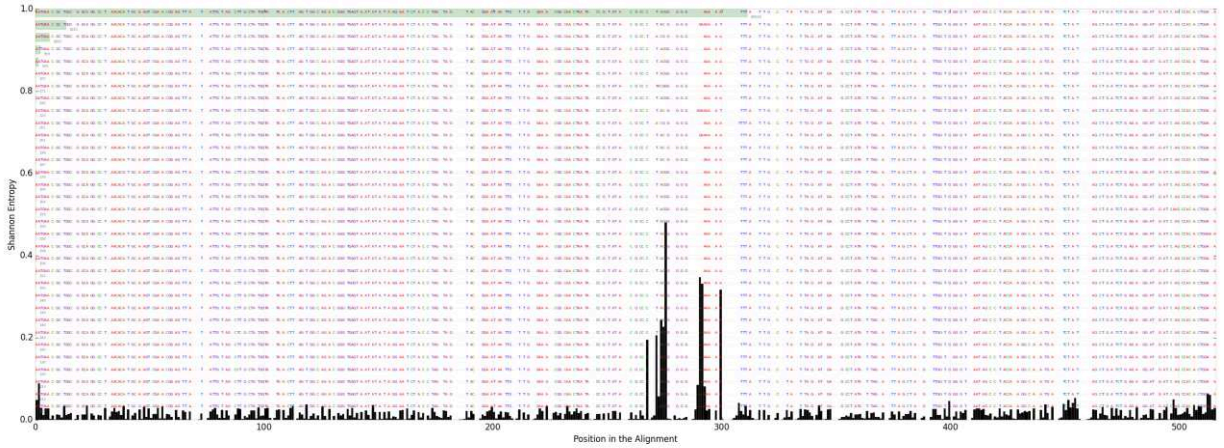

Figure S4. Entropy analysis output showing the nucleotide positions with high entropy values present in homopolymer regions.

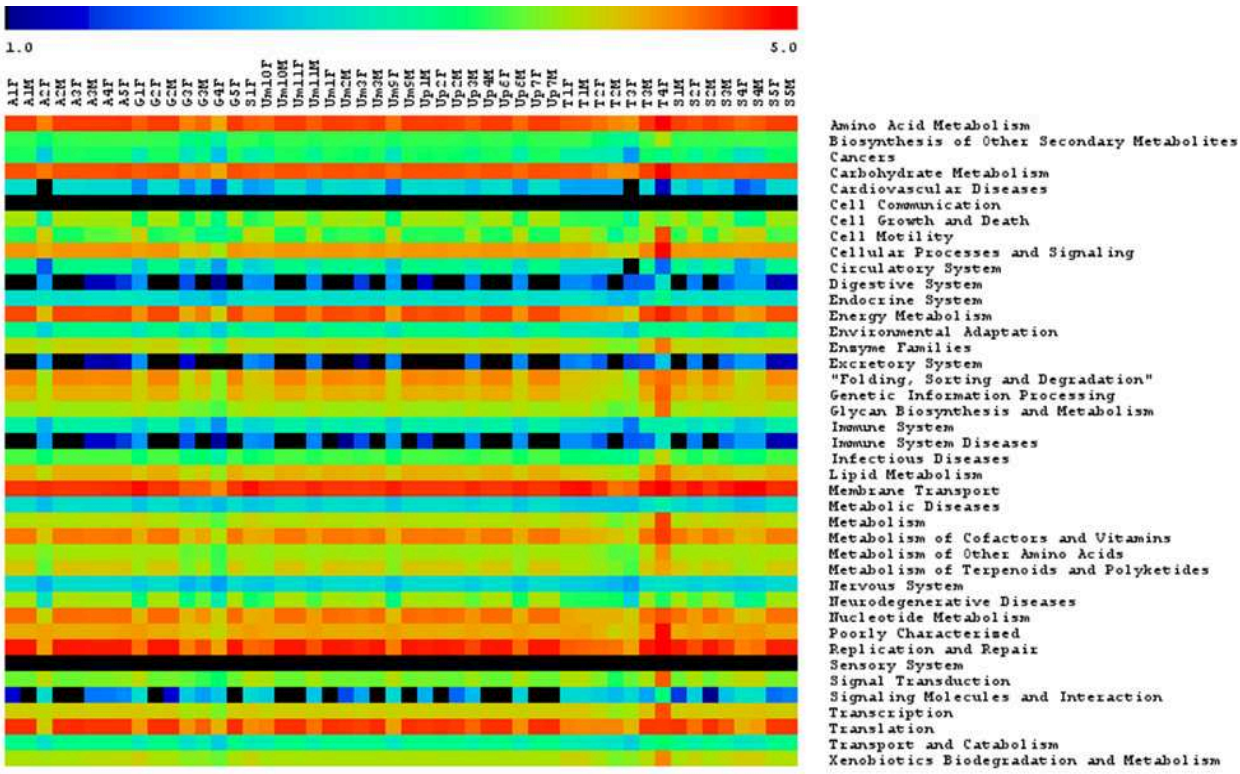

Figure S5. Heatmap in log scale depicting the PICRUST-inferred relative gene abundance in the bacterial metagenome of *H.abietis* of different sampling locations in the presence of *Wolbachia*. Warm colors represent high abundances, cold colors represent low abundance and black indicates absence

Table S1. Statistics of 454 pyrosequencing of *Hylobius abietis* gut microbiota after open-reference OTU picking for downstream analyses.

Code	Location	Trap	Sex	Pooled guts	Raw reads	Reads after quality trimming[1]	Without *Wolbachia*	Rarefied [2]
A1F	Asa	1	Female	4	3884	3868	160	0
A1M	Asa	1	Male	5	1353	1343	6	0
A2F	Asa	2	Female	6	703	693	691	481
A2M	Asa	2	Male	5	1021	1005	0	0
A3F	Asa	3	Female	5	1650	1624	6	0
A3M	Asa	3	Male	5	3393	3360	302	0
A4F	Asa	4	Female	5	5970	5876	1232	481
A5F	Asa	5	Female	5	702	684	58	0
G1F	Gotland	1	Female	5	775	759	654	481
G1M	Gotland	1	Male	5	0	0	0	0
G2F	Gotland	2	Female	5	4456	4413	165	0
G2M	Gotland	2	Male	5	4756	4740	87	0
G3F	Gotland	3	Female	5	9013	8954	7182	481
G3M	Gotland	3	Male	5	2488	2484	1419	481
G4F	Gotland	4	Female	5	872	872	798	481
G4M	Gotland	4	Male	5	34	0	0	0
G5F	Gotland	5	Female	4	7283	7257	14	0
S1F	Spain	1	Female	5	1612	1574	1256	481
S1M	Spain	1	Male	5	1340	1326	135	0
S2F	Spain	2	Female	4	7177	7122	4294	481
S2M	Spain	2	Male	6	806	802	16	0
S3F	Spain	3	Female	4	535	517	503	481
S3M	Spain	3	Male	6	1371	1365	653	481
S4F	Spain	4	Female	6	2748	2729	2547	481
S4M	Spain	4	Male	3	31913	31744	27856	481
S5F	Spain	5	Female	6	13238	13171	1619	481
S5M	Spain	5	Male	4	1088	1078	188	0
T1F	Germany	1	Female	5	9449	9330	6702	481
T1M	Germany	1	Male	5	19797	19716	14523	481
T2F	Germany	2	Female	5	1087	1081	759	481
T2M	Germany	2	Male	5	6039	6019	4349	481
T3F	Germany	3	Female	5	1341	1339	1333	481
T3M	Germany	3	Male	5	9725	9674	1515	481
T4F	Germany	4	Female	5	1560	1524	1482	481
T4M	Germany	4	Male	5	0	0	0	0
Um1F	Umeå	1	Female	5	863	853	10	0
Um2M	Umeå	2	Both	3	4093	4029	457	0
Um3F	Umeå	3	Female	3	5697	5584	1606	481

Um3M	Umeå	3	Male	5	615	611	0	0
Um9F	Umeå	9	Female	5	2817	2728	2391	481
Um9M	Umeå	9	Male	5	1165	1155	28	0
Um10F	Umeå	10	Female	4	3264	3209	2205	481
Um10M	Umeå	10	Male	7	1199	1193	2	0
Um11F	Umeå	11	Female	3	1662	1650	24	0
Um11M	Umeå	11	Male	5	1019	1017	590	481
Up1M	Uppsala	1	Male	7	4494	4279	680	481
Up2F	Uppsala	2	Female	3	8363	8278	289	0
Up2M	Uppsala	2	Male	6	12168	12009	1478	481
Up3M	Uppsala	3	Male	7	2990	2813	1353	481
Up4M	Uppsala	4	Male	9	7038	6988	181	0
Up6F	Uppsala	6	Female	4	1135	1131	6	0
Up6M	Uppsala	6	Male	4	4784	4720	3802	481
Up7F	Uppsala	7	Female	3	6274	6204	160	0
Up7M	Uppsala	7	Male	6	5236	5212	38	0
OTUs					359	259	231	162
Total reads					234055	231706	97804	13468
Average					4334.4	4290.9	1811.2	481.0
Standard deviation					5410.2	5380.7	4303.0	0.0

[1] After removal of chimeras, samples with less than 500 reads and chloroplast sequences

[2] After removal of samples with less than 400 reads and rarefied the OTU table to a depth of 481 reads per sample

Table S2. Sequences used for the meta-analysis of Curculionidae-associated bacteria.

Beetle species	Sampling method	Sequences	Accession numbers	Diet	Plant	Tissue type	References
Dendroctonus ponderosae	DGGE/Cloning	12	JF810915-JF810926	Bark	Conifers	Adults	Adams *et al.* 2013
Dendroctonus frontalis	Cloning	19	DQ314782-DQ314800	Bark	Conifers	Adults	Vasanthakumar *et al.* 2006
		22	DQ316909-DQ316930			Larvae	
		17	DQ321537-DQ321653				
Dendroctonus valens		9	FJ811882-FJ11890	Bark	Conifers	Adults	Morales-Jimenez *et al.* 2009
	DGGE/Cloning	19	GQ423599-GQ423617	Bark	Conifers	Adults	Adams *et al.* 2010
Dendroctonus rhizophagus	DGGE/Cloning	14	JN712149-JN712155, JN712162, JN712164, JN712165, JN712167, JN712169, JN712173-JN712175	Bark	Conifers	Adults Pupa Larvae	Morales-Jimenez *et al.* 2012
Ips pini	Cloning	73	DQ836634-DDQ836706	Bark	Conifers	Larvae	Delalibera *et al.* 2007
Rhynchophorus ferrugineus	TGGE/Cloning	42	KC896717-KC896758	Bark/palms	Palms	Larvae	Tagliavia *et al.* 2014
Otiorhynchus salicicola	454	15	JN563736-JN563750	Roots	Ornamental	Larvae	Hirsch *et al.* 2012
Otiorhynchus rugosostriatus	454	9	JN563751-JN563759	Roots	Ornamental	Larvae	Hirsch *et al.* 2012
Otiorhynchus sulcatus	454	3	JN563760-JN563762	Roots	Ornamental	Larvae	Hirsch *et al.* 2012
Otiorhynchus armadillo	454	22	JN563763-JN563784	Roots	Ornamental	Larvae	Hirsch *et al.* 2012

Table S3. P-values of the different statistical analyses performed for assessing differences in microbial community profiles among countries, locations, and sexes, based on different distance matrixes.

	ANOSIM				
	All countries			Only Sweden	
Distance matrix	Country	Location	Sex	Location	Sex
Unifrac (unweigh.)	0.006	0.006	0.623	0.183	0.516
Sorensen	0.002	0.001	0.345	0.182	0.24
Bray-curtis	0.001	0.001	0.275	0.149	0.211
Jaccard	0.002	0.001	0.236	0.157	0.233

	ADONIS				
	All countries			Only Sweden	
Distance matrix	Country	Location	Sex	Location	Sex
Unifrac (unweigh.)	0.001	0.001	0.773	0.093	0.473
Sorensen	0.001	0.001	0.725	0.194	0.374
Bray-curtis	0.001	0.001	0.738	0.22	0.452
Jaccard	0.001	0.001	0.753	0.152	0.376

	Discriminant analysis				
	All countries (all OTUS)			Only Sweden (all OTUS)	
	Cuntry	Location	Sex	Location	Sex
Wilk´s lambda	0.48	0.116	0.88	0.295	0.671
P value	0.028	0.001	0.546	0.531	0.408

Table S4. Oligotyping statistics.

	OTU 59	OTU 136	OTU 150	OTU 164
Number of entropy positions selected	6	4	22	6
Sequences analyzed	4152	3170	1374	4124
Number of initial samples	23	28	25	25
Number of Initial oligotypes	41	17	41	27
Number of oligotypes after QF	15	5	6	10
Final number of sequences	3906	3006	1168	3933
% of sequences used	94.08%	94.83%	85.01%	95.37%
Number of samples eliminated	1	1	4	0

Table S5. P-values of the different statistical analyses performed for assessing differences in oligotype profiles in different OTUs among countries, locations, and sexes, based on a Bray-Curtis similarity matrix.

	OTU 59	OTU 136	OTU 150	OTU 164
Sex	0.52	0.6	0.632	0.861
Location	0.02	0.1	0.07	0.016
Country	0.03	0.04	0.038	0.09

3.10.1 Supplementary References

Adams AS, Adams SM, Currie CR, Gillette NE, Raffa KF (2010) Geographic variation in bacterial communities associated with the red turpentine beetle (Coleoptera: Curculionidae). *Environmental Entomology* 39, 406-414.

Adams AS, Aylward FO, Adams SM, *et al.* (2013) Mountain pine beetles colonizing cistorical and naive host trees are associated with a bacterial community highly enriched in genes contributing to terpene metabolism. *Applied and Environmental Microbiology* 79, 3468-3475.

Delalibera I, Vasanthakumar A, Burwitz BJ, *et al.* (2007) Composition of the bacterial community in the gut of the pine engraver, *Ips pini* (Say) (Coleoptera) colonizing red pine. *Symbiosis* 43, 97-104.

Morales-Jimenez J, Zuniga G, Ramirez-Saad HC, Hernandez-Rodriguez C (2012) Gut-associated bacteria throughout the life cycle of the bark beetle *Dendroctonus rhizophagus* Thomas and Bright (Curculionidae: Scolytinae) and their cellulolytic activities. *Microbial Ecology* 64, 268-278.

Morales-Jimenez J, Zuniga G, Villa-Tanaca L, Hernandez-Rodriguez C (2009) Bacterial community and nitrogen fixation in the red turpentine beetle, *Dendroctonus valens* LeConte (Coleoptera: Curculionidae: Scolytinae). *Microbial Ecology* 58, 879-891.

Hirsch J, Strohmeier S, Pfannkuchen M, Reineke A (2012) Assessment of bacterial endosymbiont diversity in *Otiorhynchus* spp. (Coleoptera: Curculionidae) larvae using a multitag 454 pyrosequencing approach. *BMC Microbiology* 12.

Tagliavia M, Messina E, Manachini B, Cappello S, Quatrini P (2014) The gut microbiota of larvae of *Rhynchophorus ferrugineus* Oliver (Coleoptera: Curculionidae). *BMC Microbiology* 14.

Vasanthakumar A, Delalibera I, Handelsman J, *et al.* (2006) Characterization of gut-associated bacteria in larvae and adults of the southern pine beetle, *Dendroctonus frontalis* Zimmermann. Environmental *Entomology* 35, 1710-1717.

CHAPTER IV

BACTERIAL AND FUNGAL SYMBIONTS OF PARASITIC *DENDROCTONUS* BARK BEETLES

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4.1. ABSTRACT

Bark beetles (Curculionidae: Scolytinae) are one of the most species-rich herbivorous insect groups with many shifts in ecology and host-plant use, which may be mediated by their bacterial and fungal symbionts. While symbionts are well studied in economically important, tree-killing species, little is known about parasitic species whose broods develop in living trees. Here, using culture-dependent and independent methods, we provide a comprehensive overview of the associated bacteria, yeasts and filamentous fungi of the parasitic *Dendroctonus micans*, *D. punctatus* and *D. valens,* and compare them to those of other tree-inhabiting insects. Despite inhabiting different geographical regions and/or host trees, the three species showed similar microbial communities. Enterobacteria were the most prevalent bacteria, in particular *Rahnella*, *Pantoea* and *Ewingella*, in addition to *Streptomyces*. Likewise, the yeasts *Candida/Cyberlindnera* were the most prominent fungi. All these microorganisms are widespread among tree-inhabiting insects with various ecologies, but their high prevalence overall might indicate a beneficial role such as detoxification of tree defenses, diet supplementation or protection against pathogens. As such, our results enable comparisons of symbiont communities of parasitic bark beetles with those of other beetles, and will contribute to our understanding of how microbial symbioses facilitate dietary shifts in insects.

4.2. INTRODUCTION

Herbivory by insects is evolutionary derived (Labandeira and Sepkoski 1993) and highly successful given the accelerated rates of speciation after switching to herbivorous diet (Mitter *et al.* 1988; Farrell 1998). Microbial symbionts play a major role in this transition, because many plant materials are nutritionally imbalanced for herbivorous insects (Watanabe and Tokuda 2010). Symbiotic bacteria and fungi can provide their insect hosts with essential capabilities for synthesizing nutrients, overcoming plant defenses, digesting lignocellulosic plant tissue and detoxifying plant defensive chemicals (Douglas 2009; Gibson and Hunter 2010). In addition, microbial symbionts may also help herbivorous insects to protect themselves against microbial competitors, pathogens and/or predators (Florez *et al.* 2015).

Bark beetles (Coleoptera: Curculionidae: Scolytinae) are one of the most successful herbivorous insect groups with at least 6000 known species worldwide (Knízek and Beaver 2004). Most of these attack woody plant tissues and studied species harbor diverse bacterial and fungal symbionts. As for other insects, these symbionts offer different services to their hosts, which researchers are just beginning to understand (Six 2013; Hofstetter *et al.* 2015). The tremendous diversity of bark beetle feeding habits and ecologies as well as the repeated evolutionary switches between these make bark beetle–microbe symbioses a relevant model for studying the function and evolution of insect symbionts in relation to host ecology (Six and Klepzig 2004; Six 2012; Kirkendall *et al.* 2015).

The relatively small genus *Dendroctonus* (19 described species) is the best studied bark beetle group today, and fungal as well as bacterial symbionts have been studied in several species (Six and Klepzig 2004; Davis 2014; Hofstetter *et al.* 2015). They are fascinating from an evolutionary point of view, because of their diversity of host-use strategies which are expected to strongly shape symbiont communities (Six and Klepzig 2004; Six 2012). *Dendroctonus* species have been classified in three groups depending on their host use: (i) aggressive tree killers (hence-forth termed aggressive beetles; e.g. *Dendroctonus frontalis*, *D. ponderosae*), (ii) parasites on living trees that do not kill their host (henceforth termed parasitic beetles; e.g. *D. micans, D. punctatus*) and (iii) early successional saprophages on dead or dying trees (henceforth termed saprophagous beetles; e.g. *D. approximatus*, although it has also been reported to attack healthy trees) (Lindgren and Raffa 2013; Six and Bracewell 2015). As already observed by Lindgren and Raffa (2013), *D. valens* is somehow unusual, with a flexible status. In its native range, it would usually rather qualify as an early saprophage on the stumps of freshly cut hosts, or on lightning-struck trees, but it can also be parasitic on living trees (Smith 1971). Quite contrastingly, the North American *D. valens* aggressively attacks and kills living trees in China, where it was introduced and became epidemic under climatic conditions stressful for the host trees (Sun *et al.* 2013). On the whole, this species should thus rather be seen as near parasitic.

Although all *Dendroctonus* spp. invariably attack conifer hosts, these aggressive, parasitic and saprophagous species are faced with completely different challenges according to the types of hosts they colonize, and are therefore expected to profit from symbionts in different ways (Six and Klepzig 2004; Lindgren and Raffa 2013). Aggressive beetles, for example, would profit from symbionts that help them to overcome the tree defenses and to deal with declining phloem quality (Six and Klepzig 2004; Bleiker and Six 2007). By contrast, parasitic beetles are not expected to associate with symbionts that seriously harm the host tree, but instead with ones that help them to detoxify constitutive and continuously induced chemical tree defenses. The fact that only symbionts of aggressive beetles are well studied, however, currently limits our ability to develop a theoretical framework describing how host-use impacts symbiont communities and their functional roles in *Dendroctonus* (Six and Bracewell 2015).

The filamentous fungi are the best studied symbionts of *Dendroctonus*. Many aggressive beetles are obligately dependent on fungal symbionts, which they transmit from their natal brood system to the new

host in highly selective spore-carrying organs, called mycangia, on the exoskeleton (Six 2003). These mycangial fungi supplement the beetles' phloem diet with additional nitrogen and sterols (Ayres *et al.* 2000), and in some cases may help beetles to overcome tree defenses (Six and Klepzig 2004); although the latter role has been questioned recently (Six and Wingfield 2011). Many filamentous fungi can be isolated from bark beetles, but only those found with a high prevalence (notably in mycangia) can be regarded as obligate mutualists. The limited information available on parasitic bark beetles suggests that they do not have mycangia and do not engage in obligate mutualisms with filamentous fungi (Six and Bracewell 2015).

Yeasts are ubiquitous associates of bark beetles, but their functional role for the host is not well understood. In *Dendroctonus*, they are often isolated at high rates, but only few are host-specific (Rivera *et al.* 2009). They can play a beneficial role in beetle pheromone communication (Zhao *et al.* 2015) and some enhance the growth of fungal mutualists while suppressing fungal competitors and/or entomopathogens in vitro (Adams *et al.* 2008; Davis *et al.* 2013). Others negatively affect beetles by attracting natural enemies or by producing toxic chemicals (Boone *et al.* 2008). Again, in *Dendroctonus*, yeast communities are mostly described for the aggressive beetles.

Bacterial symbionts of *Dendroctonus* are ubiquitous in beetle guts, mycangia and galleries. Many taxa could provide nutritional benefits to the beetles by accessing sugars from complex polymers, recycling nitrogen from beetle excretions or fixing atmospheric nitrogen (Engel and Moran 2013; Morales-Jimenez *et al.* 2013). Other bacterial symbionts can detoxify host tree defensive compounds (Adams *et al.* 2013; Boone *et al.* 2013), defend their hosts against microbial competitors or pathogens (Cardoza *et al.* 2006; Scott *et al.* 2008) and/or facilitate the growth of the fungal mutualists (Adams *et al.* 2008). Generally, *Dendroctonus* species host relatively species-poor bacterial communities compared to other insects; this is likely due to the protected nature of the habitat and the toxicity of the phloem (Franceschi *et al.* 2005; Six and Bracewell 2015). Many symbionts of *Dendroctonus* are also known as symbionts of plants, likely because of their pre-adaptations to plant defenses. Whether indeed plant symbionts play a role in the beetles' success and what proportion of the insects' symbionts is picked up anew from the tree every generation is currently unknown (despite recent attempts, e.g. Mason *et al.* 2016).

Dendroctonus micans (Kugelann) and D*. punctatus* LeConte are two parasitic species with similar ecologies; *D. valens* LeConte seems to have a different ecology (see above). However, all three can complete their entire life cycle within living hosts: *D. micans* mainly on spruce in Europe and Asia, *D. punctatus* on spruce in Northern USA, Canada and Alaska, and *D. valens* on pines in Mexico, USA and Southern Canada (Smith 1971; Wood 1982; Gregoire 1988; Furniss 1995). A single female (*D. micans and D. punctatus*) or pair (*D. valens)* bores a gallery in the inner bark, often near ground level, and lays batches of eggs. Larvae feed gregariously in a communal chamber where frass is accumulated and pupation occurs. Emerging adults either mate with siblings or, in the case of *D. valens*, after dispersal. Microbial symbionts have been studied in several populations of *D. valens* (Six and Klepzig 2004; Rivera *et al.* 2009; Adams *et al.* 2010). Surveys of *D. micans*' symbionts were limited to culturable bacteria in Turkish populations and ophiostomatoid fungi in one French population (Lieutier *et al.* 1992; Yilmaz *et al.* 2006). Non-culturable bacteria and other fungi have not been investigated in *D. micans*, and the microbial communities of *D. punctatus* remain completely unknown.

Here we describe an extensive survey of bacterial and fungal symbionts of *D. valens, D. micans* and *D. punctatus* using a common set of culture-dependent and independent techniques (454 pyrosequencing). Our first objective is to characterize symbiont communities of three parasitic or near-parasitic *Dendroctonus* species. We further investigate whether these communities change during insect development or lab rearing, and if microorganisms could be taken up from the surrounding phloem. Second, we discuss how symbiont functional differences may be related to respective insect ecologies by

comparing our microbial communities of parasitic bark beetles with published data on the communities of aggressive beetles.

4.3. Material and Methods

4.3.1. Beetle collection

Field insects

Mature adults and second and third instar larvae (L2-3) were sampled. *Dendroctonus micans* were collected on living trees in Commana, Merdrignac and Scrignac, Brittany, France (48◦ 24 09.60 N 3◦ 56 20.02 W; 48◦ 27 51.71 N 3◦ 38 14.97 W), in April–October 2012 and August 2013. Phloem samples were jointly taken 1 cm left or right from the edge of *D. micans* larval chambers in order to document the bacterial symbionts in the beetles' environment. *Dendroctonus* punctatus were collected on living trees west of Prince George, British Columbia, Canada (53◦ 42 44 N 122◦ 52 26 W; 53◦ 43 04.57 N 122◦ 53 07.01 W; 53◦ 51 06 N 123◦ 12 21 W) in July 2012 and July 2013. *Dendroctonus valens* were collected on fresh stumps and lightning-struck trees east of Redding, California, USA (40◦ 30 28.80 N 121◦ 51 52.77 W; 40◦ 32 43 N 121◦ 46 50 W; 40◦ 43 03.78 N 121◦ 59 27.04 W) in July 2012 and July 2013.

Laboratory insects

Only mature adults were sampled from the laboratory reared populations. *Dendroctonus punctatus* specimens were collected on a living tree north of Kamloops, British Columbia, Canada (51◦ 04 29 N 120◦ 19 52 W) in June 2011, and were reared until the second generation in the LUBIES quarantine room. *Dendroctonus valens* specimens were collected on fresh stumps in the province of Shanxi, China in 2007, and were reared until the 17th generation. As *D. micans* undergoes a reproductive diapause, laboratory adults could not be obtained for this species.

4.3.2. Culture-independent bacterial community analysis

DNA extraction, amplification and sequencing

Samples were stored in 70% ethanol at –80◦ C until analysis. Insect and phloem samples were taken out of the ethanol and dried under a sterile hood. Individual samples (whole insects or phloem) were flash frozen with liquid nitrogen and homogenized with a pestle. DNA extraction was conducted using the Epicentre MasterPure DNA Purification kit (Madison, Wisconsin, USA) in accordance with the manufacturer's instructions, including a treatment with lysozyme to break up Gram-positive bacterial cell walls. After DNA extraction, identical volumes of six individuals (except n = 4 for *D. micans* field larvae and n = 5 for D. punctatus field adults) or four phloem pieces (each 8 × 2 × 2 mm) were pooled per group for bacterial tag-encoded FLX amplicon pyrosequencing (bTEFAP). Pooled DNA samples were sent to 'Molecular Research Laboratory' (Shallowater, TX, USA) for bTEFAP with 16S rRNA primers Gray28F forward 5-GAGTTTGATCNTGGCTCA-3 and Gray519R reverse 5 -GTNTTACNGCGGCKGCTG-3 (Sun, Wolcott and Dowd 2011). A sequencing library was generated through one-step PCR with 30 cycles, using a mixture of HotStar and HotStar HiFidelity Taq polymerases (Qiagen, Hilden, Germany). Sequencing extended from Gray28F, using a Roche 454 FLX instrument with Titanium reagents and procedures at Molecular Research Labo-ratory (http://mrdnalab.com/).

454 sequencing data analysis

QIIME (i386 1.3.03) was used for analysis of the 454 sequencing data (Caporaso *et al.* 2010). All low-quality reads (minimum average quality cut-off = 25) were removed, as well as sequences shorter than 200 bp and longer than 600 bp. No errors in the barcode but one mismatch and one ambiguous base per sequence were allowed. USEARCH6.1 (QIIME) was used to identify and discard potential chimeras. The remaining reads were analyzed using a multistep operational taxonomic unit (OTU)-picking strategy with the *cdhit* (Li and Godzik 2006) algorithm set at 97% similarity cut-off. The representative sequences per OTU were determined by picking the most abundant sequence, and were aligned to the Greengenes core set (http://greengenes.lbl.gov/) with PyNast using a minimum sequence identity of 75%. Taxonomy was assigned using the *uclust* classifier and only sequences with more than 0.8 confidence were selected for further analyses. Finally, an OTU table was generated with the absolute and relative abundances of bacterial phylotypes within the samples, which is visualized in a heat map constructed with MultiExperiment Viewer 4.9.0 (Saeed *et al.* 2003). Chloroplast and mitochondria sequences were manually removed from the OTU table.

Alpha- and beta-diversity indices were calculated in QIIME based on the OTU table of absolute and relative abundances per sample. Rarefaction curves were generated by subsampling the OTU table with step increments of 10 sequences and 100 iterations to check for adequate sampling depth. Weighted and unweighted UniFrac distances were subjected to principal coordinates analyses (PCoA) to assess clustering of bacterial communities according to insect species, instar, phloem and laboratory-reared versus field samples. ANOSIM discriminant analyses between these groups were performed in QIIME using the unweighted and weighted UniFrac principal coordinates.

Phylogenetic placement of Dendroctonus microbiota

OTUs from the three sampled *Dendroctonus* spp. were compared with the bacterial communities of other bark beetle studies that used culture-independent methods. Our data set on *Dendroctonus*-associated bacteria was expanded by bacterial symbiont sequences of tree-inhabiting insect host species belonging to four genera (308 sequences from GenBank; Table S1, Supporting Information; see also Berasategui *et al.* 2016). All sequences were aligned using SINA 1.2.11 (Pruesse, Peplies and Glockner 2012), imported into ARB 5.5 (Ludwig *et al.* 2004) and then mapped onto the 16S SILVA rRNA database (which currently includes 597 607 curated bacterial sequences). Two to three OTUs, neighboring our OTUs, were then picked up from the SILVA database to construct a tree with our sequences and the quality checked bacterial sequences from SILVA (total 551 sequences; Table S1, Supporting Information). The quality of the alignment for each OTU sequence was manually checked and corrected. A total of 32 of the initial 182 OTUs that were imported in ARB did not fit among the 16S SILVA sequences and were excluded from our tree. The final tree was constructed with the remaining 150 OTUs in FastTree 2.1 using the GTR model (Price *et al.* 2010) and was edited in MEGA 6 (Tamura *et al.* 2011).

4.3.3. Culture-dependent microbiota analysis

Bacterial and fungal isolation

Live insects (n = 6) were individually surface-sterilized in 70% ethanol, rinsed in distilled water and crushed as a whole in 500 µL phosphate buffer solution with a Retsch MM301 grinder and beads (Haan, Germany). The supernatant was serially diluted 10-fold in 10 mM MgSO4 (up to 10^{-8}). Dilutions were plated (n = 2) on potato dextrose agar (PDA), yeast extract malt agar (YEMA) and nitrogen-depleted

medium described in Emtiazi, Pooyan and Shamalnasab (2007) to detect potential nitrogen-fixing bacteria. Plates were incubated at 30◦ C for optimal growth (1 to 14 days), and colony forming units (CFU) were counted based on plated dilutions. Pure cultures of all morphotypes were obtained and preserved in glycerol at −80◦ C. Yeasts were discriminated from bacteria microscopically.

DNA extraction, amplification and sequencing

Typical 2–3 mm diameter colonies of bacteria and yeasts were transferred to a cell lysis solution (67 mM Tris-HCl pH 8.8, 16.6 mM (NH4)2SO4, 5 mM b-mercaptoethanol, 6.7 mM MgCl2.6H2O, 6.7 µM EDTA pH 8.0, 1.7 µM SDS), while filamentous fungi were grown on cellophane and ground in liquid nitrogen for DNA ex-traction using the MasterPure DNA Purification kit (Epicentre).

Bacterial 16S amplification

For bacterial isolates, the 16S rRNA gene was amplified with primers fD1 (5 -AGAGTTTGATCCTGGCTCAG-3) and rP2 (5 - ACGGCTACCTTGTTACGACTT-3) (Weisburg *et al.* 1991) using the following PCR conditions: 94◦ C for 3 min; 35 cycles of 94◦ C for 40 s, 60◦ C for 1 min, 72◦ C for 1 min and a final extension of 72◦ C for 4 min. PCR was conducted with 25 U mL^{-1} TopTaq DNA polymerase and 1x TopTaq PCR buffer (Qiagen), 0.2 mM of each dNTP, 0.2 µM of each primer and typically 2.3-5 ng μL^{-1} of template DNA. PCR products were purified using 1.54 U μL^{-1} Exonuclease I (Epicentre) and 0.15 U μL^{-1} FastAP Thermosensitive Alkaline Phosphatase (ThermoFisher Scientific, Erembodegem, Belgium), and sequenced with the BigDye Terminator v3.1 Cycle Sequencing Kit on a 3730 DNA Analyzer (Applied Biosystems, Foster City, California, USA) with the same primers and additionally the primer R1087 (5 - CTCGTTGCGGCACTTAACCC-3) for the 16S rDNA.

Fungal 18S and ITS amplification

For fungal isolates, two genetic markers were sequenced. First, the 18S rRNA gene was amplified with the primers the primers NS1 (5 -GTAGTCATATGCTTGTCTC-3) and NS4 (5 -CTTCCGTCAATTYCTTTAAG-3) (White *et al.* 1990) using the following PCR conditions: 94◦ C for 30 s; 35 cycles of 94◦ C for 30 s, 55◦ C for 45 s, 72◦ C for 1 min; and 72◦ C for 7 min. Second, the ITS region was amplified with the primers ITS1 (5-TCCGTAGGTGAACCTGCGG-3) and ITS4 (5-TCCTCCGCTTATTGATATGC-3) (White *et al.* 1990) with PCR conditions as follows: 92◦ C for 2 min; 41 cycles of 95◦ C for 30 s, 55◦ C for 30 s, 72◦ C for 1 min; 72◦ C for 8 min (Six *et al.* 2009). PCR and sequencing mixtures were the same as for bacteria, except that primer couples NS1–NS4 and ITS1–ITS4 were used.

BLAST

Consensus sequences were assembled in Geneious Pro 4.8.5 (Biomatters, Auckland, New Zealand) and blasted against the GenBank database. Taxa were considered identified at the species level with a match level of at least 97% identity, at the genus level with a match of at least 95%, and were considered unidentified below 95%. Phylogenetic relationships among isolated strains and closest BLASTn matches were constructed using MOLE-BLAST with the neighbor-joining method.

4.4. RESULTS

4.4.1. Culture-independent bacterial community analysis

Our metagenomic analysis of *Dendroctonus micans, D. punctatus* and *D. valens* field adults, larvae, phloem and laboratory-reared adults yielded a total of 169 107 sequences (after quality trimming), which were clustered into 182 OTUs. OTUs were assigned to the level of 56 different genera, 57 families, 32 orders and 20 classes of bacteria. In the relative abundance heat map of the 50 most abundant OTUs (Fig. 1), OTUs 230 (*Luteibacter),* 124 (*Pseudomonas)*, 251 (*Ralstonia*), 216 (*Erwinia*), 50 (other unidentified

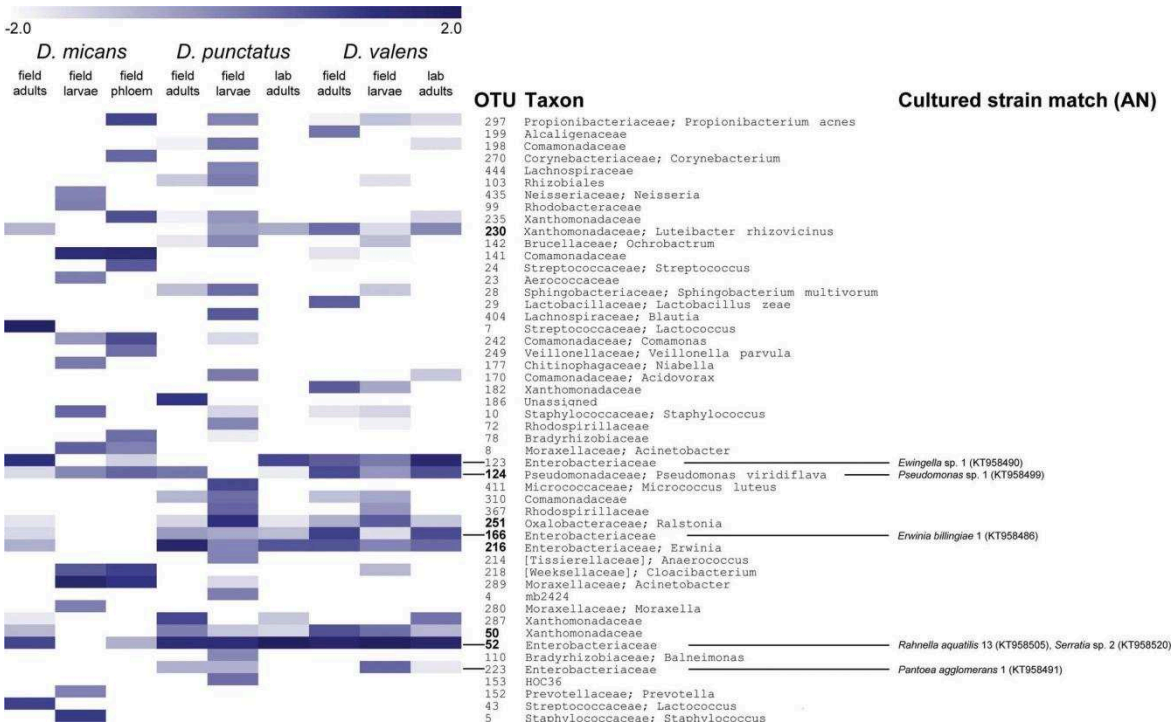

Figure 1. Relative abundance heat map (log-transformed, white = absence, dark blue = high) of the 50 most abundant pyrosequenced bacterial OTUs (based on the cumulative relative abundance among all groups) in *D. micans, D. punctatus* and *D. valens* field and laboratory larvae, adults and distant phloem. Bold OTUs are the most conserved among all samples of the three beetle species, lines denote OTUs with corresponding isolates (i.e. identity > 99.3%) and accession numbers (AN) that we gained by culturing on PDA, YEMA and/or nitrogen-depleted media.

Xanthomonadaceae), 166 and in particular 52 (both other unidentified Enterobacteriaceae) were the most conserved among the three studied parasitic or near-parasitic bark beetles. Within *D. punctatus*, the prominent *Erwinia* (OTU 216) was almost absent from the second generation of lab-reared adults while an unidentified enterobacterium (OTU 52, close to *Rahnella* spp.) was 3.5 times as abundant as in field adults. Similarly, the rare enterobacterial OTU 123 (also close to *Rahnella* spp.) was more than 10 times as abundant and many less abundant OTUs were completely absent in the 17th generation lab-reared *D. valens,* in comparison with the field population.

Except *D. micans* field adults, whose community was predominated by Firmicutes (*Lactococcus*), all other samples mainly harbored Gammaproteobacteria (Fig. 2). Depending on the sample, these were mainly *Acinetobacter* (only in *D. micans* field larvae and nearby phloem) or *Erwinia* and other unidentified

genera in all others. Betaproteobacteria (*Ralstonia* and other Comamonadaceae) were common only in *D. punctatus* and *D. micans* field larvae and nearby phloem. Rarefaction analyses demonstrated sufficient sampling depth for all our samples (Fig. S1, Supporting Information). Field-collected *D. micans* hosted less bacterial OTUs (25 ± 5 OTUs, mean ± SD) than *D. punctatus* (57 ± 23) and *D. valens (*46 ± 6) (Table 1). Field-collected adults and larvae were more species rich (45 ± 3) than those reared in the laboratory (25 ± 9).

Table 1. Chao1 alpha-diversity indices of pyrosequenced bacterial OTUs (at equal sampling depth of 1762 sequences) in *D. micans, D. punctatus* and *D. valens* field and laboratory larvae, adults and distant phloem.

Species	Origin	Instar	Total N of OTUs
Dendroctonus micans	Field	Adults	30
	Field	Larvae	20
	Field	Phloem	24
Dendroctonus punctatus	Field	Adults	42
	Field	Larvae	72
	Lab	Adults	15
Dendroctonus valens	Field	Adults	48
	Field	Larvae	45
	Lab	Adults	34

!

The weighted PCoA components explained a total of 82.8% of the variance in the OTUs' relative abundance (Fig. S2, Supporting Information). All instars of *D. punctatus* and *D. valens* clustered together, except *D. punctatus* field larvae, which were a bit closer to another cluster composed of *D. micans* field larvae and nearby phloem. *Dendroctonus micans* field adults were distant from all other clusters. The unweighted PCoA components explained 45.7% of variance (Fig. S3, Supporting Information). There was no effect of species (ANOSIM $P = 0.71$) and origin ($P = 0.42$) on the bacterial communities based on unweighted UniFrac. However, a significant effect of stages ($P = 0.023$) on bacterial communities was found. This analysis needs to be interpreted with care since (i) abundance of OTUs lead to important differences between the weighted and unweighted PCoA plots (Figs S2 and S3, Supporting Information), and (ii) ANOSIM on weighted UniFrac values only detected a tendency for clustering for species ($P = 0.056$) but not for origin ($P = 0.39$) and stage ($P = 0.39$).

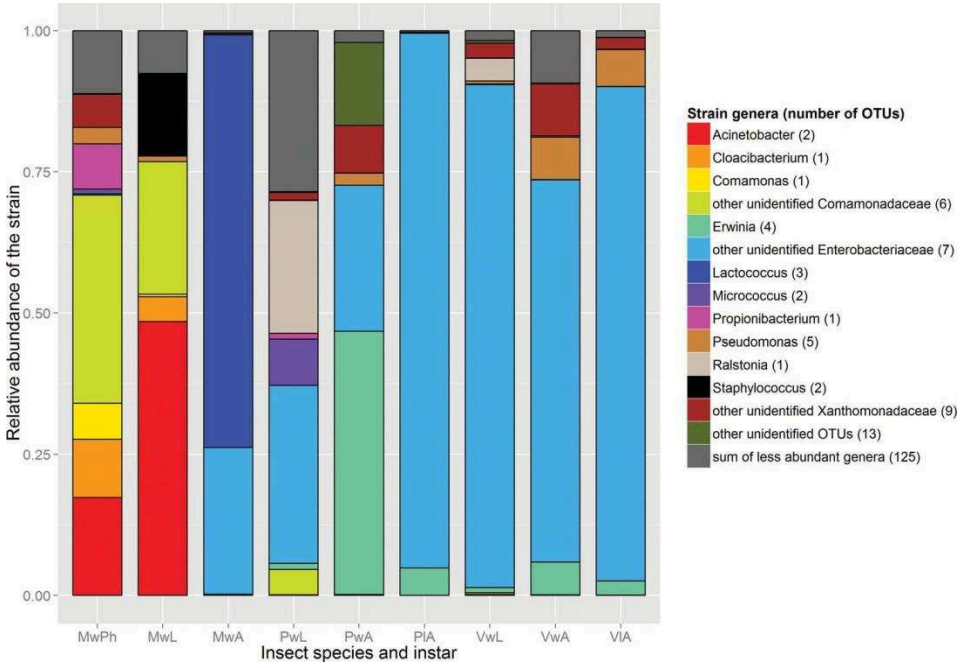

Figure 2. Relative abundance of pyrosequenced bacterial genera in *D. micans* (M), *D. punctatus* (P) and *D. valens* (V) field (w) and laboratory (l) larvae (L), adults (A) and distant phloem (Ph).

The phylogenetic position of the three sampled *Dendroctonus* species' OTUs is shown in comparison with the bacterial community of other bark- and wood-inhabiting insects, and bacteria isolated from the environment (Table S1; Figs. 3 and S4, S5, Supporting Information). The *Dendroctonus* OTUs were close neighbors of bacteria from 16 different host insects belonging to 11 genera, in particular the bark beetles *Ips pini, D. frontalis* and to a lesser extend *D. rhizophagus*. There was an overlap of OTUs with previous studies on *D. valens*; for *D. micans* and *D. punctatus*, no culture-independent data on bacterial communities were available for comparison. Nevertheless, most OTUs were closely related to bacteria that were originally not isolated from insects, and were rather free-living bacteria.

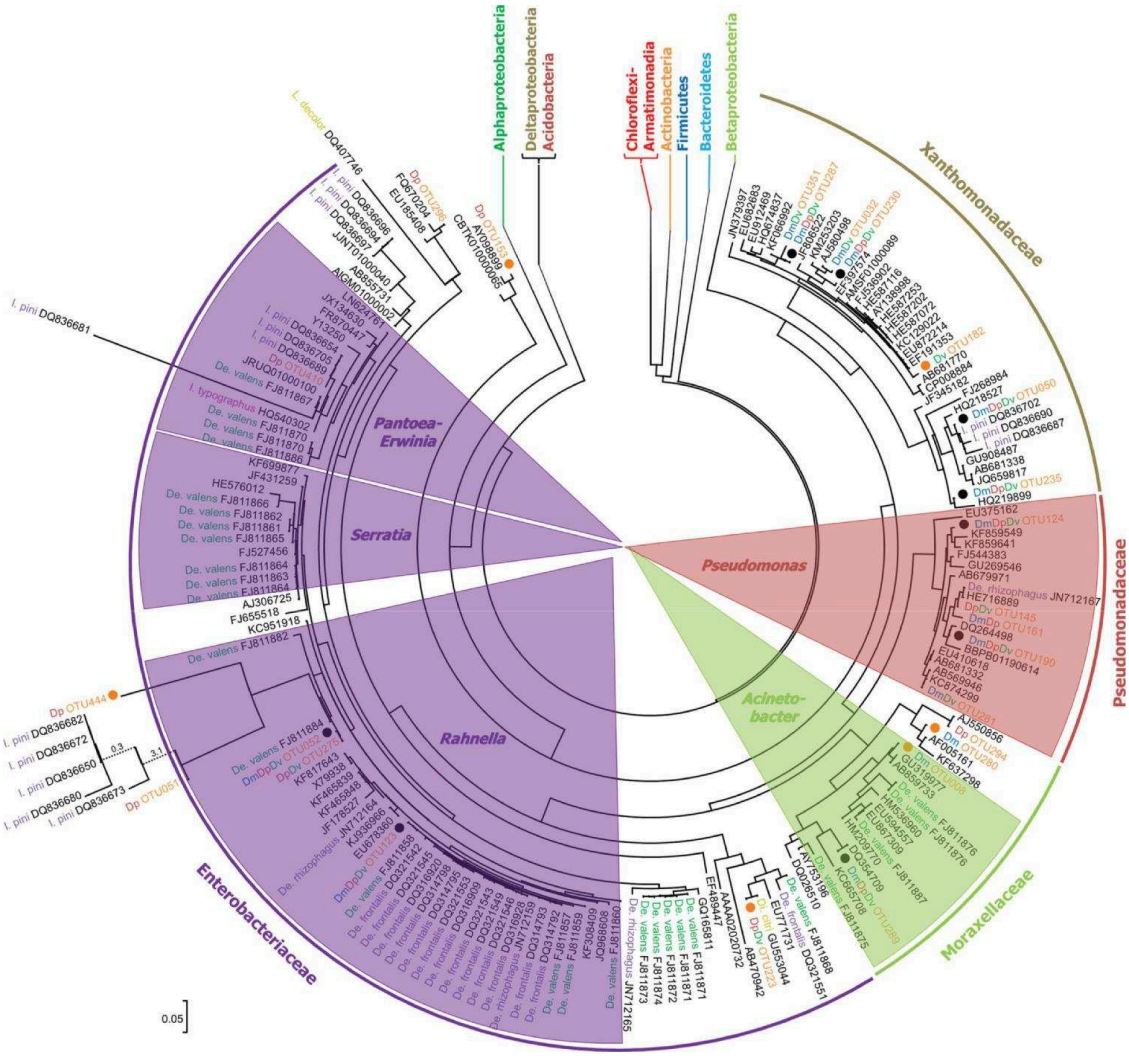

Figure 3. Phylogenetic tree of representative sequences of pyrosequenced bacterial OTUs associated with *D. micans, D. punctatus* and *D. valens* in relation to the symbionts of other insects and environmental bacteria. Only Gammaproteobacteria are shown, all other taxa are collapsed. Taxa from this study are highlighted in orange; the 50 most abundant OTUs are indicated by an orange dot; OTUs present in at least one instar of all three species are indicated by a black dot. Black accession numbers are provided for taxa from other studies. The insect host, when known, is attached to the taxa, where Dm = *D. micans* (in blue), Dp = *D. punctatus* (in red), Dv = *D. valens* (in green), A. = *Apis*, Ce. = *Cephalotes*, Cr. = *Cryptopone*, De. = *Dendroctonus*, Di. = *Diaphorina*, I. = *Ips*, L. = *Liposcelis*, M. = *Moechotypa*, R. = *Reticulitermes*, S. = *Saccharococcus* and X. = *Xylosandrus*. Spruce-feeding insects (except *D. micans* and *D. punctatus*) are in pink, pine-feeding insects (except D. *val*ens) are in violet, and other insect hosts are in yellow. There is no mention of insect host for bacteria isolated from the environment. The length of plain branches represents the genetic distance according to the scale, dotted branches could not be plotted in full size and have their true genetic distance indicated.

4.4.2. Culture-dependent microbiota analysis

A total of 116 bacterial and 83 fungal isolates were cultured from *D. micans, D. punctatus* and *D. valens* field adults and larvae, including 37 and 27 identified strains, 16 and 18 species, and 9 and 7 genera, respectively (Table S2, Supporting Information).

Bacterial community

The mean bacterial population was 1.7 × 107 CFUs/adult and 6.0 × 105 CFUs/larva (Figs S6 and S7, Supporting Information; Table 2). *Rahnella aquatilis* was the most widespread bacterium; it consistently inhabited all instars of *D. micans* and *D. valens* (≥ 67% of prevalence). Similarly, *Pantoea cedenensis* was present in at least 50% of *D. punctatus* larvae and all instars of *D. valens*. Other very prevalent taxa were *Ewingella* sp. in 83% of *D. punctatus* adults, *Streptomyces lienomycini* in 67% of *D. punctatus* larvae, *Pa. agglomerans* in 67% of *D. valens* adults and *Rouxiella* sp. in 67% of *D. micans* adults.

Fungal community

The mean fungal population was 4.9 × 107 CFUs/adult and 2.9 × 104 CFUs/larva. Overall, yeasts were the most abundant fungi (Figs 4, S8 and S9, Supporting Information; Table 2), especially *Candida*/*Cyberlindnera* spp. They were isolated from all three insect species and dominated particularly in *D. micans* and in *D. valens* field adults (≥67% of samples). These yeasts were less abundant in *D. punctatus* field larvae, where *Hypocrea muroiana*/ *Trichoderma viride* (83%) and *Pichia bispora*/ *Yeast* sp. 5 (67%) were found instead. *Penicillium*/ *Talaromyces* spp. were the most abundant filamentous fungi and were only absent in *D. valens* field larvae. Despite being ubiquitous, these were inconsistent associates among the insects, except *Penicillium* sp. 2/ *Talaromyces variabilis* in *D. micans* field adults (50%).

4.4.3. Complementarity of culture-dependent and independent analyses

Bacterial 16S sequences from cultured strains and pyrosequenced OTUs were aligned for potential matches between both methods. Seven OTUs (Fig. 1; Table S2, Supporting Information) corresponded with six different Enterobacteriaceae species (*Erwinia billingiae*, E*. typographi, Ewingella* sp. 1, *Pa. agglomerans*, *Ra. aquatilis, Serratia* sp. 2) and two Pseudomonadaceae species (*Pseudomonas* sp. 1 and 2), accounting for a total of 101 305 sequences (59.9% of sequences from all OTUs).

Table 2. Identity and prevalence (% of sampled individuals) of bacterial and fungal isolates that we gained by culturing on PDA, YEMA and nitrogen-depleted media from *D. micans*, *D. punctatus* and *D. valens* field larvae and adults. Asterisk indicates isolates that were able to grow on nitrogen-depleted medium, = = denotes synonyms, / indicates assignments equally closely related.

Prevalence (%)	*Dendroctonus micans*		*Dendroctonus punctatus*		*Dendroctonus valens*	
Identified strains	Larvae	Adults	Larvae	Adults	Larvae	Adults
Bacteria						
Enterobacter sp.	0	0	0	0	0	33
Erwinia billingiae	0	0	0	0	0	17
Erwinia sp.	0	0	0	0	17	0
Erwinia typographi	0	0	0	33*	33*	0
Ewingella sp.	0	0	0	83*	33	0
Pantoea agglomerans	0	17*	0	0	0	67*
Pantoea cedenensis	0	0	67*	33*	50*	50*
Pseudomonas sp. *1*	0	0	0	0	17*	33
Pseudomonas sp. *2*	0	0	0	0	17*	0
Rahnella aquatilis	67*	100*	17*	0	83*	67
Rouxiella sp.	0	67*	0	0	0	0
Serratia liquefaciens	0	0	0	17	0	0
Serratia marcescens	0	0	0	0	17*	0
Serratia sp. *1*	0	0	0	0	0	33*
Serratia sp. *2*	0	17*	0	0	0	0
Streptomyces lienomycini	0	0	67	17	0	0
Fungi						
Acanthophysium cerussatum/Stereum gausapatum	17*	0	0	17*	0	0
Candida piceae	33	0	33	17	33	67
other unidentified *Candida* spp.	83*	33*	0	17*	0	67*
Candida sp. *1/Candida* sp. *3*	33*	17	0	0	0	17
Candida sp. *2/C. fructus*	50*	67*	0	17*	0	0
Cyberlindnera americana/Candida sp. *3*	33	17	0	0	33	17*
Hypocrea muroiana/Trichoderma viride	0	0	83*	0	0	0
Penicillium charlesii/P. pulvis	0	0	17*	0	0	17
Penicillium chrysogenum	0	17*	0	0	0	0
Penicillium corylophilum	0	17*	0	0	0	0
Penicillium decumbens	17	0	0	0	0	0
Penicillium purpurogenum = = *Talaromyces purpurogenus*	0	0	17	0	0	0
other unidentified *Penicillium* spp.	17*	0	17	17*	0	0
Penicillium sp. *1/Talaromyces radicus*	0	0	17	0	0	0
Penicillium sp. *2/Talaromyces variabilis*	17*	50*	0	0	0	17*
Pichia bispora/Yeast sp. *5*	0	0	67	0	0	0
Fungus sp. *8*	0	17	0	0	0	0
Yeast sp. *7*	0	0	0	0	0	17

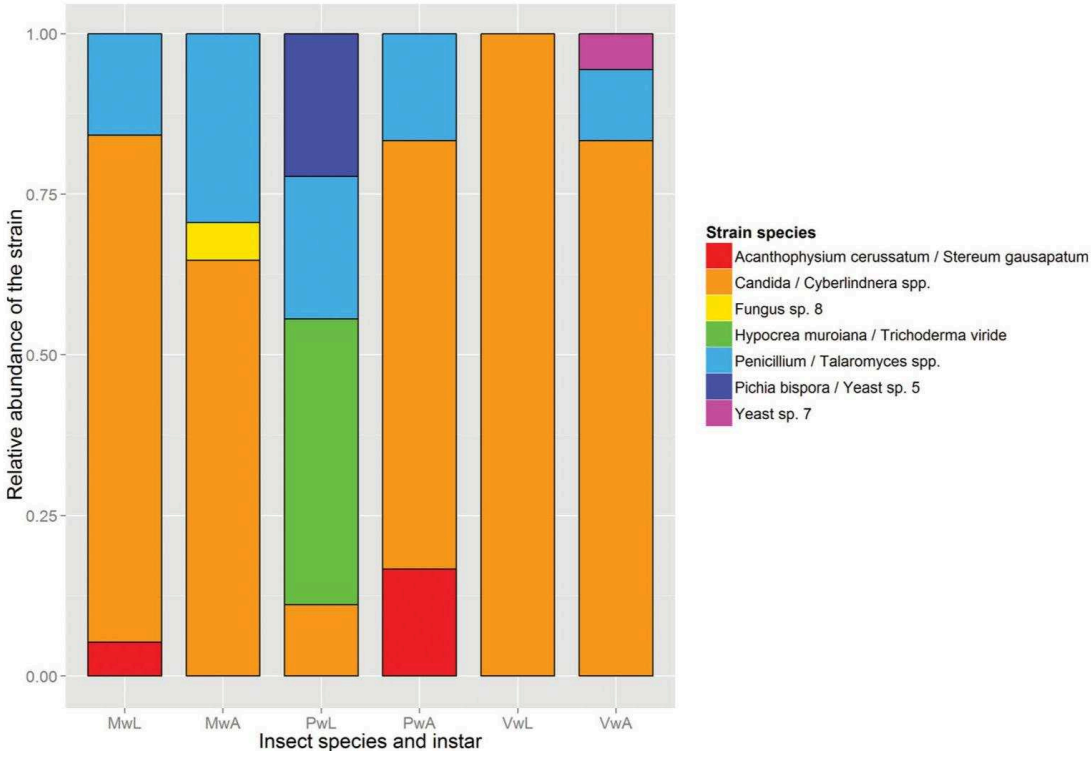

Figure 4. Relative abundance of cultured fungal taxa in *D. micans* (M), *D. punctatus* (P) and *D. valens* (V) field (w) larvae (L) and adults (A).

4.5. DISCUSSION

In this study, we characterized the bacterial and fungal communities associated with three bark beetles, *Dendroctonus micans, D. punctatus* and *D. valens,* which have a similar parasitic or near-parasitic ecology. Despite inhabiting distinct geographical regions and/or different host trees, these insects shared a large proportion of their microbial symbionts: (1) Enterobacteriaceae (Gammaproteobacteria) was the dominant bacterial family with the most prevalent species *Rahnella aquatilis*, *Pantoea* ce*denensis*, *Ewingella* sp., *Pa. agglomerans* and *Rouxiella* sp. (although this one is very close to *Ra. aquatilis*). Enterobacteria, especially the aforementioned genera and species, are often reported as symbionts of bark- and wood-boring insects (Morales-Jimenez *et al.* 2009, 2012; Hu *et al.* 2013; Aylward *et al.* 2014; Xu *et al.* 2015; Berasategui *et al.* 2016; Mason *et al.* 2016). (2) *Streptomyces lienomycini* was commonly isolated from *D. punctatus*. *Streptomyces* bacteria are frequent associates of North American bark beetles (Hulcr *et al.* 2011). (3) Yeasts were the most abundant fungi, especially *Candida/Cyberlindnera* spp. in *D. micans and D. valens, whereas D. punctatus* also commonly harbored *Pichia bispora*. Likewise, these yeasts are frequent symbionts of bark and ambrosia beetles (Rivera *et al.* 2009; Davis 2014; Lou *et al.* 2014).

All these prevalent microorganisms are close relatives of common, free-living bacteria (Figs 3, S4 and S5, Supporting Information) and yeasts. *Rahnella aquatilis*, *Ewingella* spp., *Pa. agglomerans* as well as *Candida* spp. are regularly isolated from soil, water and also from plants as epiphytes and endophytes (Weinthal *et al.* 2007; Winder, Macey and Cortese 2010; Ribeiro and Cardoso 2012; Hu *et al.* 2015). The latter means that they likely occur in the vicinity of bark- and wood-boring beetles, which may explain why they are so frequently isolated from these insects (Colman *et al.* 2012). As a striking illustration of endophyte intake, *D. micans* larvae housed bacteria very similar to nearby phloem, with prominent *Acinetobacter* and *Comamonadaceae*. Our phloem samples from just *D. micans*, however, have low explanatory power in this respect and it remains unknown which microorganisms are present in the phloem already before bark beetles establish their galleries there (Mason *et al.* 2016). Nevertheless, even if their associated microbes can occur in the environment, this does not exclude possible functional roles for the performance of *D. micans, D. punctatus* and *D. valens.* Facultative symbionts can make important contributions to host fitness, even when they are acquired from the environment (Kikuchi *et al.* 2007; Engel and Moran 2013).

Overall, the bacterial community associated with (near) parasitic bark beetles does not seem very constant. Unlike *D. punctatus*, the bacterial communities of *D. valens* and *D. micans* have already been studied before and greatly vary geographically (Yilmaz *et al.* 2006; Morales-Jimenez *et al.* 2009; Adams *et al.* 2010; Xu *et al.* 2015; this study). In *D. valens*, the bacterial community from the Californian population we sampled (where *D. valens* qualifies as a saprophagous-parasitic species) was much more distant to the one from the Northern USA (Adams *et al.* 2010) than to the ones from Mexico (Morales-Jimenez *et al.* 2009) or from invasive populations in China (although *D. valens* behaves aggressively there; Xu *et al.* 2015). Despite some geographical variation, *Rahnella* and *Pantoea* were consistent symbionts throughout all four studies. In the bacterial community of *D. micans*, there is only a small overlap (mainly *Serratia*) in the identified taxa from France (this study) and from Turkey (Yilmaz *et al.* 2006). By contrast to these parasitic beetles, the bacterial community of the aggressive *D. ponderosae* is much more stable (Adams *et al.* 2013). Variable bacterial communities could be related to rather opportunistic, loose symbiotic associations, with little effect for their host (Engel and Moran 2013). However, symbiont redundancy, where different microorganisms play similar roles (Six 2012), could also explain this pattern. Throughout the developmental stages, the bacterial community of *D. valens* was very constant, but the ones of both D*. micans* and *D. punctatus* were quite distinct between field-collected adults and larvae. Several non-exclusive hypotheses could explain such community changes during the developmental stages: (i) successive molts disrupt the gut bacterial flora (Engel and Moran 2013); (ii) adults and larvae feed on different parts of the phloem, which may be of different quality (e.g. in terms of nutrition and defense compounds; Franceschi *et al.* 2005); (iii) larvae and adults select different symbionts according to their needs; (iv) temporal turnover of the symbionts (unlike *D. punctatus, D. micans* larvae and adults were collected in the same place but at different times).

The bacterial species richness was much lower in laboratory-reared adults in comparison with field insects. In addition, we observed differences in the relative abundance of several bacterial OTUs. These results are similar to those of Meeus *et al.* (2015) who observed that *Bombus terrestris* reared indoors had a subset of the bacterial community compared to field insects. It is possible that the differences in the bacterial communities result from the rearing conditions and could, in turn, affect the beetles' physiology. In many insects, the gut community is strongly influenced by the diet (Engel and Moran 2013). In our laboratory conditions, insects were reared on fresh logs and phloem sandwiches that are likely to differ from a standing, living tree (i.e. moisture content, toxic plant compounds and general food quality; Klepzig and Six 2004; Six and Klepzig 2004) and which different microorganisms may exploit. Alternatively, in the specific case of *D. valens*, the community change between field and lab adults may be related to the different collection places.

The fungal community of *D. micans, D. punctatus* and *D. valens* was dominated by widespread environmental yeasts and some ubiquitous filamentous fungi. Not a single ophiostomatoid fungus was isolated. Most aggressive *Dendroctonus* spp. are tightly associated with these fungi (Six and Bracewell 2015). In its native range, *D. valens* behaves like a saprophage and parasite, and is occasionally associated with ophiostomatoid fungi, although with high variability of prevalence (Six and Klepzig 2004, Sun *et al.* 2013). In China, where *D. valens* aggressively attacks living trees, it is associated with phytopathogenic strains of *Leptographium procerum* and other ophiostomatoid species, however, with a very low prevalence (Lu *et al.* 2009, 2010; Sun *et al.* 2013). These insect–fungus interactions are very complex: *D. valens* could benefit from symbiotic nutritional supplementation or help in overcoming the tree defenses (Sun *et al.* 2013), but feeding experiments demonstrated that *L. procerum*, and other ophiostomatoid associates of *D. valens*, compete with the insects for polysaccharides in the phloem and trigger an immune response in the beetles, which resulted in lower weight gain in larvae (Shi *et al.* 2012; Wang *et al.* 2013). Such a competition for the available resources might be one reason explaining why true parasitic beetles are not consistently associated with ophiostomatoid fungi specifically, or filamentous fungi in general, which in this case are regarded as opportunistic associates (Lieutier *et al.* 1992; Raffa, *et al.* 1993; Six and Bracewell 2015). Another reason may be the secondary metabolites of living trees that are toxic to both insects and fungi (Raffa and Smalley 1995; Franceschi *et al.* 2005; Krokene 2015), but to which parasitic beetles evolved a high resistance (Everaerts *et al.* 1988). Last, it is also counteradaptive for parasitic beetles that derive large benefits from their life in living trees (i.e. a stable environment protecting them from generalist natural enemies and from competitors) to inoculate their galleries with fungi that have been shown (i) to trigger higher induced tree defenses, which harm the beetles (Raffa and Smalley 1995), or even (ii) kill the tree. Future surveys should focus on the prevalence and possible roles of ophiostomatoid associates of parasitic bark beetles, which could in turn improve our knowledge on those of aggressive bark beetles.

Parasitic bark beetles live in and feed on phloem that is loaded with constitutive and induced plant defensive compounds. Therefore, symbionts that are able to assist in detoxification should be very beneficial to the beetles, like *Ra. aquatilis* (Boone *et al.* 2013; Xu *et al.* 2015) and *Candida*/*Cyberlindnera* spp. (Rivera *et al.* 2009; Lou *et al.* 2014) which we both isolated at high rates in our study. By-products of such microorganisms may also play a role in the intra- or interspecific chemical communication of the beetles (Boone *et al.* 2008; Zhao *et al.* 2015). Bark beetles could also benefit from symbionts by protection and improvement of their nutritional niche. Living phloem is lacking many nutrients, like nitrogen sources and sterols (Merrill and Cowling 1966; Bentz and Six 2006). In this context, associated microorganisms could provide a significant advantage to parasitic bark beetles. First, several prevalent bacteria were isolated on nitrogen-depleted medium in this study and are known as nitrogen fixing (diazotroph): *Ra. aquatilis* (Vasanthakumar *et al.* 2006; Morales-Jimenez *et al.* 2009) and *Pa. agglomerans* (Bridges 1981). Moreover, NifH genes were amplified from the microbial community of D*. micans, D*. *punctatus* and *D. valens* (Dohet and Biedermann, unpublished data). Likewise, *Ra. aquatilis* is also reported as nitrogen recycling (uricolytic; Morales-Jimenez *et al.* 2013). Second, cellulolytic microorganisms were detected in preliminary tests on Congo red agar (Dohet, unpublished data), which may enrich the beetles' phloem diet with additional free carbohydrates. Furthermore, microorganisms such as the isolated actinomycetes *Micrococcus luteus* or *Streptomyces* spp. could shape beetles' microbial communities by producing antibiotics as shown for strains of these taxa in *D. rufipennis* and *D. frontalis*, respectively (Cardoza *et al.* 2006; Scott *et al.* 2008).

Our study suggests that parasitic bark beetles lack consistent association with highly phytopathogenic fungi but could benefit, at the same time, from other symbionts. It also discloses striking differences between parasitic bark beetles, two of which (*D. micans* and *D. punctatus*) almost never kill their hosts and are inconsistently found associated with ophiostomatoid fungi (*D. micans*; Lieutier *et al.* 1992) or have never been studied so far in this respect (*D. punctatus*). On the other hand, some near-parasitic bark beetles, like *D. valens*, sometimes massively kill trees and harbor ophiostomatoid fungi with a low

prevalence in this case (Lu *et al.* 2009). *Dendroctonus murrayanae*, which also qualifies as parasitic, has been observed killing trees (Wood 1982) and is consistently associated with ophiostomatoid fungi (Six *et al.* 2011). In summary, we believe that these important differences in the ecology of parasitic beetles call for a substantial revision of this whole group, distinguishing true parasites from near parasites.

This is the first comprehensive characterization of the bacterial and fungal symbionts of the (near) parasitic bark beetles *D. micans*, *D. punctatus* and *D. valens*, using a combination of culture-dependent and independent methods. Many of the close relatives of the identified taxa have been previously characterized as free-living microorganisms, but others are known as symbionts of plants, bark beetles and other wood-boring insects. Parasitic and near-parasitic bark beetles could benefit from these symbionts in various ways, through detoxification of tree defenses, diet supplementation and/or protection against pathogens. Due to the high variability of associated bacteria and fungi, future studies should sample all developmental stages of parasitic bark beetles more intensively from various populations and at different times to identify a potential core microbiota, spatiotemporal patterns and their concrete effects on their hosts. This sampling should also take the surrounding phloem and wood into account, which will help to clarify which microbes are present within the tree beforehand and are not inoculated by the beetles. Such studies will help to understand the roles of symbionts in shifts of ecologies and hosts in bark beetles, which are a great model for the development of a broad theoretical framework on the function and evolution of bacterial and fungal symbionts in insects as a whole.

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4.8. CONFLICT OF INTEREST.

None declared.

4.9. REFERENCES

Adams AS, Adams SM, Currie CR *et al.* (2010) Geographic variation in bacterial communities associated with the red turpentine beetle (Coleoptera: Curculionidae). *Environmental Entomology,* 39:406–14.

Adams AS, Aylward FO, Adams SM *et al.* (2013) Mountain pine beetles colonizing historical and naive host trees are associated with a bacterial community highly enriched in genes contributing to terpene metabolism. *Applied and Environmental Microbiology,* 79:3468–75.

Adams AS, Six DL, Adams SM *et al.* 2008) In vitro interactions between yeasts and bacteria and the fungal symbionts of the mountain pine beetle (*Dendroctonus ponderosae*). *Microbial Ecology,* ;56:460–66.

Aylward FO, Suen G, Biedermann PHW *et al.* (2014) Convergent bacterial microbiotas in the fungal agricultural systems of insects. *mBio*, 5:e02077.

Ayres MP, Wilkens RT, Ruel JJ *et al.* (2000) Nitrogen budgets of phloem-feeding bark beetles with and without symbiotic fungi. *Ecology*, 81:2198–10.

Bentz BJ, Six DL (2006) Ergosterol content of fungi associated with *Dendroctonus ponderosae* and *Dendroctonus rufipennis* (Coleoptera: Curculionidae, Scolytinae). *Annals of the Entomological Society of America,* 99: 189–94.

Berasategui A, Axelsson K, Schmidt A *et al.* (2016) The gut microbiota of the pine weevil is similar across Europe and resembles that of other conifer-feeding beetles. *Molecular Ecology*, DOI:10.1111/mec.13702.

Bleiker KP, Six DL. (2007) Dietary benefits of fungal associates to an eruptive herbivore: potential implications of multiple associates on host population dynamics. *Environmental Entomology*, 36:1384–96.

Boone CK, Keefover-Ring K, Mapes AC *et al. (2013)* Bacteria associated with a tree-killing insect reduce concentrations of plant defense compounds. *Journal of Chemical Ecology,*39:1003–06.

Boone CK, Six DL, Zheng Y *et al.* (2008) Parasitoids and dipteran predators exploit volatiles from microbial symbionts to locate bark beetles. *Environmental Entomology,*37:150–61.

Bridges J (1981) Nitrogen-fixing bacteria associated with bark beetles. *Microbial Ecology,* 7:131–7.

Caporaso JG, Kuczynski J, Stombaugh J *et al.* (2010) QIIME allows analysis of high-throughput community sequencing data. *Nat* ure *Methods*, 7:335–6.

Cardoza YJ, Klepzig KD, Raffa KF (2006) Bacteria in oral secretions of an endophytic insect inhibit antagonistic fungi. *Ecological Entomology,* 31:636–45.

Colman DR, Toolson EC, Takacs-Vesbach CD (2012) Do diet and taxonomy influence insect gut bacterial communities? *Molecular Ecology,*21:5124–37.

Davis TS (2014) The ecology of yeasts in the bark beetle holobiont: a century of research revisited. *Microbial Ecology*, 69:723–32.

Davis TS, Crippen TL, Hofstetter RW *et al.* (2013) Microbial volatile emissions as insect semiochemicals *Journal of Chemical Ecology*, 39: 840–59.

Douglas A (2009) The microbial dimension in insect nutritional ecology. *Functional Ecology*, 23:38–47.

Emtiazi G, Pooyan M, Shamalnasab M (2007) Cellulase activities in nitrogen fixing *Paenibacillus* isolated from soil in N-free media. *World Journal of Agricultural Sciences,* 3:602–8.

Engel P, Moran NA (2013) The gut microbiota of insects - diversity in structure and function. *FEMS Microbiol Reviews,* 37:699–735.

Everaerts C, Gregoire JC, Merlin J (1988) The toxicity of Norway spruce monoterpenes to two bark beetle species and their associates. In: Mattson WJ, Levieux J, Bernard-Dagan C (eds). Mechanism of Woody Plant Defenses Against Insects. New York: Springer, 335–44.

Farrell BD (1998) 'Inordinate fondness' explained: why are there so many beetles? *Science*, 281:555–59.

Florez´ LV, Biedermann PHW, Engl T *et al.* (2015) Defensive symbioses of animals with prokaryotic and eukaryotic microorganisms. *Natural Product Reports*, 32:904–36.

Franceschi VR, Krokene P, Christiansen E *et al. (*2005) Anatomical and chemical defenses of conifer bark against bark beetles and other pests. *New Phytologist*, 167:353–75.

Furniss MM (1995) Biology of *Dendroctonus punctatus* (Coleoptera: Scolytidae). *Annals of the Entomological Society of America,* 88:173–82.

Gibson CM, Hunter MS (2010) Extraordinarily widespread and fantastically complex: comparative biology of endosymbiotic bacterial and fungal mutualists of insects. *Ecology Letters*,13: 223–34.

Gregoire JC (1988) The greater European spruce beetle. In: Berryman AA (ed). Dynamics of Forest Insect Populations: Patterns, Causes, Implications. New York: Springer Science+Business Media, 455–78.

Hofstetter RW, Dinkins-Bookwalter J, Davis TS *et al.* (2015) Symbiotic associations of bark beetles. In: Vega FE, Hofstetter RW (eds). Bark Beetles. San Diego: Academic Press, 209–45.

Hu X, Li M, Chen H (2015) Community structure of gut fungi during different developmental stages of the Chinese white pine beetle (*Dendroctonus armandi*). *Scienctific Reports,* 5:8411.

Hu X, Wang C, Chen H *et al.* (2013) Differences in the structure of the gut bacteria communities in development stages of the Chinese white pine beetle (*Dendroctonus armandi*). *International Journal of Molecular Sciences,* 14:21006–20.

Hulcr J, Adams AS, Raffa K *et al.* (2011) Presence and diversity of Streptomyces in *Dendroctonus* and sympatric bark beetle galleries across North America. *Microbial Ecology,* 61:759–68.

Kikuchi Y, Hosokawa T, Fukatsu T (2007) Insect-microbe mutualism without vertical transmission: a stinkbug acquires a beneficial gut symbiont from the environment every generation. *Applied and Environmental Microbiology,* 73:4308–16.

Kirkendall LR, Biedermann PHW, Jordal BH (2015) Evolution and diversity of bark and ambrosia beetles. In: Vega FE, Hofstetter RW (eds). Bark Beetles. San Diego: Academic Press, 85–156.

Klepzig KD, Six DL (2004) Bark beetle-fungal symbiosis: context dependency in complex associations. *Symbiosis*;37: 189–205.

Knı´zekˇ M, Beaver R (2004) Taxonomy and systematics of bark and ambrosia beetles. In: Lieutier F, Day KR, Battisti A *et al.* (eds). Bark and Wood Boring Insects in Living Trees in Europe, a Synthesis. Dordrecht: Springer, 41–54.

Krokene P (2015) Conifer defense and resistance to bark beetles. In: Vega FE, Hofstetter RW (ed.). Bark Beetles. San Diego: Academic Press, 177–207.

Labandeira CC, Sepkoski JJ (1993) Insect diversity in the fossil record. *Science*, 261:310–15.

Li W, Godzik A (2006) Cd-hit: a fast program for clustering and comparing large sets of protein or nucleotide sequences. *Bioinformatics*, 22:1658–9.

Lieutier F, Vouland G, Pettinetti M *et al. (*1992) Defence reactions of Norway spruce (*Picea abies* Karst.) to artificial insertion of *Dendroctonus micans* Kug. (Col., Scolytidae). *Journal of Applied Entomology,* 114:174–86.

Lindgren BS, Raffa KF (2013) Evolution of tree killing in bark beetles (Coleoptera: Curculionidae): trade-offs between the maddening crowds and a sticky situation. *Canadian Entomologist*, 145:471– 95.

Lou QZ, Lu M, Sun JH (2014) Yeast diversity associated with invasive *Dendroctonus valens* killing *Pinus tabuliformis* in China using culturing and molecular methods. *Microbial Ecology,* 68:397– 15.

Lu M, Wingfield MJ, Gillette NE *et al.* (2010) Complex interactions among host pines and fungi vectored by an invasive bark beetle. *New Phytologist*, 187:859–66.

Lu Q, Decock C, Zhang XY *et al.* (2009) Ophiostomatoid fungi (Ascomycota) associated with *Pinus tabuliformis* infested by *Dendroctonus valens* (Coleoptera) in northern China and an assessment of their pathogenicity on mature trees. *Antonie Van Leeuwenhoek International Journal of General And Molecular Microbiology,* 96:275–93.

Ludwig W, Strunk O, Westram R *et al.* ARB: a software environment for sequence data. *Nucleic Acids Res* 2004;32:1363–71.

Mason CJ, Hanshew AS, Raffa KF (2016) Contributions by host trees and insect activity to bacterial communities in *Dendroctonus valens* (Coleoptera: Curculionidae) galleries, and their high overlap with other microbial assemblages of bark beetles. *Environmental Entomology*, 45:348–56.

Meeus I, Parmentier L, Billiet A *et al.* (2015) 16S rRNA amplicon sequencing demonstrates that indoor-reared bumblebees (*Bombus terrestris*) harbor a core subset of bacteria normally associated with the wild host. *Plos One*, 10:e0125152.

Merrill W, Cowling EB (1966) Role of nitrogen in wood deterioration: amounts and distribution of nitrogen in tree stems. *Canadian Journal of Botany*, 44:1555–80.

Mitter C, Farrell B, Wiegmann B (1988) The phylogenetic study of adaptive zones: has phytophagy promoted insect diversification? *American Naturalist,* 132:107–28.

Morales-Jimenez J, Vera-Ponce de Leon A, Garcıa-Domınguez A *et al.* (2013) Nitrogen-fixing and uricolytic bacteria associated with the gut of *Dendroctonus rhizophagus* and *Dendroctonus valens* (Curculionidae: Scolytinae). *Microbial Ecology,* 66:200–10.

Morales-Jimenez J, Zuniga G, Ramırez-Saad HC *et al.* (2012) Gut-associated bacteria throughout the life cycle of the bark beetle *Dendroctonus rhizophagus* Thomas and Bright (Curculionidae: Scolytinae) and their cellulolytic activities. *Microbial Ecology,* 64:268–78.

Morales-Jimenez J, Zuniga G, Villa-Tanaca L *et al. (*2009) Bacterial community and nitrogen fixation in the red turpentine beetle, *Dendroctonus valens* LeConte (Coleoptera: Curculionidae: Scolytinae). *Microbial Ecology,* 58:879–91.

Price MN, Dehal PS, Arkin AP (2010) FastTree 2 – Approximately maximum-likelihood trees for large alignments. *PLoS One*, 5:e9490.

Pruesse E, Peplies J, Glockner FO (2012) SINA: Accurate high-throughput multiple sequence alignment of ribosomal RNA genes. *Bioinformatics*, 28:1823–9.

Raffa KF, Phillips TW, Salom SM (1993) Strategies and mechanisms of host colonization by bark beetles. In: Schowalter TD, Filip GM (eds). Beetle-Pathogen Interactions in Conifer Forests. London: Academic Press,, 103–28.

Raffa KF, Smalley EB (1995) Interaction of pre-attack and induced monoterpene concentrations in host conifer defense against bark beetle-fungal complexes. *Oecologia*, 102:285–95.

Ribeiro CM, Cardoso EJBN (2012) Isolation, selection and characterization of root-associated growth promoting bacteria in Brazil Pine (*Araucaria angustifolia*). *Microbiological Research,* 167:69–78.

Rivera FN, Gonzalez E, Gomez Z *et al.* (2009) Gut-associated yeast in bark beetles of the genus *Dendroctonus* Erichson (Coleoptera: Curculionidae: Scolytinae). *Biological Journal of the Linnean Society,* 98:325–42.

Saeed AI, Sharov V, White J *et al.* (2003) TM4: A free, open-source system for microarray data management and analysis. *BioTechniques*, 34:374–8.

Scott JJ, Oh DC, Yuceer MC *et al.* (2008) Bacterial protection of beetle-fungus mutualism. *Science*, 322:63.

Shi ZH, Wang B, Clarke SR *et al.* (2012) Effect of associated fungi on the immunocompetence of red turpentine beetle larvae, *Dendroctonus valens* (Coleoptera: Curculionidae: Scolytinae). *Insect Science*, 19:579–84.

Six DL (2003) Bark beetle-fungus symbioses. In: Bourtzis K, Miller TA (eds). Insect Symbiosis. Boca Raton: CRC Press, 97–114.

Six DL (2012) Ecological and evolutionary determinants of bark beetle —fungus symbioses. *Insects*, 3:339–66.

Six DL (2013) The bark beetle holobiont: why microbes matter. *Journal of Chemical Ecology,* 39:989–1002.

Six DL, Bracewell R (2015) *Dendroctonus*. In: Vega FE, Hofstetter RW (ed.). Bark Beetles. San Diego: Academic Press, 305–50.

Six DL, de Beer ZW, Duong TA *et al.* (2011) Fungal associates of the lodgepole pine beetle, *Dendroctonus murrayanae*. *Antonie Van Leeuwenhoek International Journal of General And Molecular Microbiology*, 100:231–44.

Six DL, Doug Stone W, de Beer ZW *et al.* (2009) *Ambrosiella beaveri*, sp. nov., associated with an exotic ambrosia beetle, *Xylosandrus mutilatus* (Coleoptera: Curculionidae, Scolytinae), in Mississippi, USA. *Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology*, 96:17–29.

Six D, Klepzig K (2004) *Dendroctonus* bark beetles as model systems for studies on symbiosis. *Symbiosis*; 37:207–32.

Six DL, Wingfield MJ (2011) The role of phytopathogenicity in bark beetle-fungus symbioses: a challenge to the classic paradigm. *Annual Review in Entomology,* 56:255–72.

Smith RH (1971) Red Turpentine Beetle. US Department of Agriculture, Forest Service, Forest Pest Leaflet, 1–9.

Sun J, Lu M, Gillette NE *et al.* (2013) Red turpentine beetle: innocuous native becomes invasive tree killer in China. *Annual Review in Entomology*, 58:293–311.

Sun Y, Wolcott RD, Dowd SE (2011) Tag-encoded FLX amplicon pyrosequencing for the elucidation of microbial and functional gene diversity in any environment. *Methods in Molecular Biology*, 733:129–41.

Tamura K, Peterson D, Peterson N *et al.* (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular Biology and Evolution,* 28:2731–9.

Vasanthakumar A, Delalibera I, Handelsman J *et al.* (2006) Characterization of gut-associated bacteria in larvae and adults of the Southern pine beetle, *Dendroctonus frontalis* Zimmermann. *Environmental Entomology*, 35:1710–7.

Wang B, Lu M, Cheng C *et al.* (2013) Saccharide-mediated antagonistic effects of bark beetle fungal associates on larvae. *Biological Letters,* 9:20120787.

Watanabe H, Tokuda G (2010) Cellulolytic systems in insects. *Annual Review in Entomology, 55:609–32.*

Weinthal DM, Barash I, Panijel M *et al.* (2007) Distribution and replication of the pathogenicity plasmid pPATH in diverse populations of the gall-forming bacterium *Pantoea agglomerans*. *Applied and Envrionmental Microbiology,* 73:7552–61.

Weisburg WG, Barns SM, Pelletier DA *et al.* (1991) 16S ribosomal DNA amplification for phylogenetic study. *Journal of Bacteriology,* 173:697– 703.

White TJ, Bruns T, Lee S *et al.* (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ *et al.* (eds). PCR Protocols: A Guide to Methods and Applications. San Diego: Academic Press, 315–22.

Winder RS, Macey DE, Cortese J (2010) Dominant bacteria associated with broods of mountain pine beetle, *Dendroctonus ponderosae* (Coleoptera: Curculionidae, Scolytinae). *Journal of the Entomological Society of British Columbia,* 107:43–56.

Wood SL (1982) The Bark and Ambrosia Beetles of North America (Coleoptera: Scolytidae), a Taxonomic Monograph. Provo: Brigham Young University.

Xu L, Lou Q, Cheng C *et al.* (*2015)* Gut-associated bacteria of *Dendroctonus valens* and their involvement in verbenone production. *Microbial Ecology, 70:1012–23.*

Yilmaz H, Sezen K, Kati H *et al.* (2006) The first study on the bacterial flora of the European spruce bark beetle, *Dendroctonus micans* (Coleoptera: Scolytidae). *Biologia* (*Bratisl*), 61:679–86.

Zhao T, Axelsson K, Krokene P *et al.* (2015) Fungal symbionts of the spruce bark beetle synthesize the beetle aggregation pheromone 2-methyl-3-buten-2-ol. *Journal of Chemical Ecology*, 41: 848–52.

4.10. SUPPLEMENTARY MATERIAL

Supplementary tables are available at FEMSEC online.

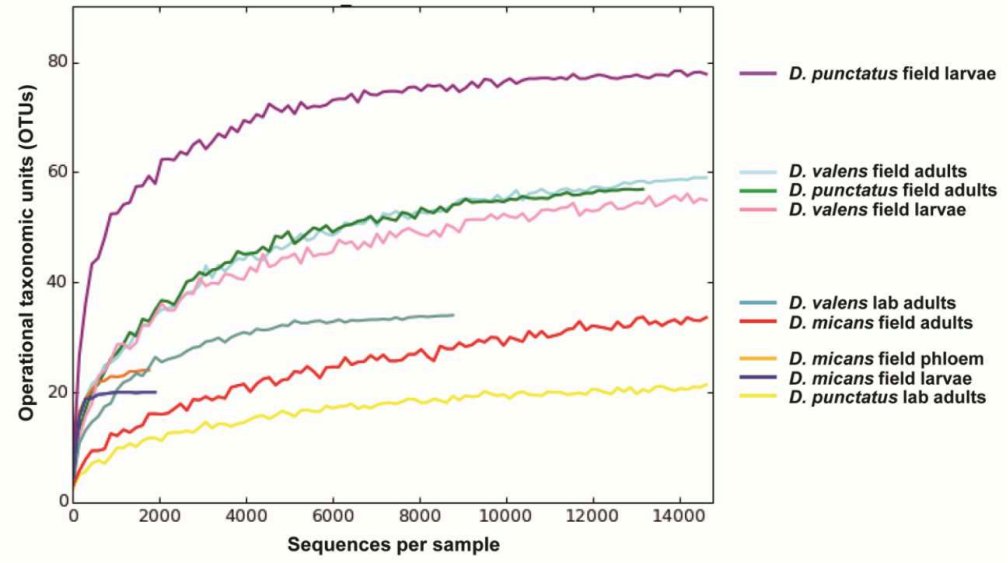

Fig. S1. Rarefaction curves of pyrosequenced bacterial OTUs in *D. micans, D. punctatus and D. valens* field and laboratory larvae, adults and distant phloem, at the level of 3% difference between sequences.

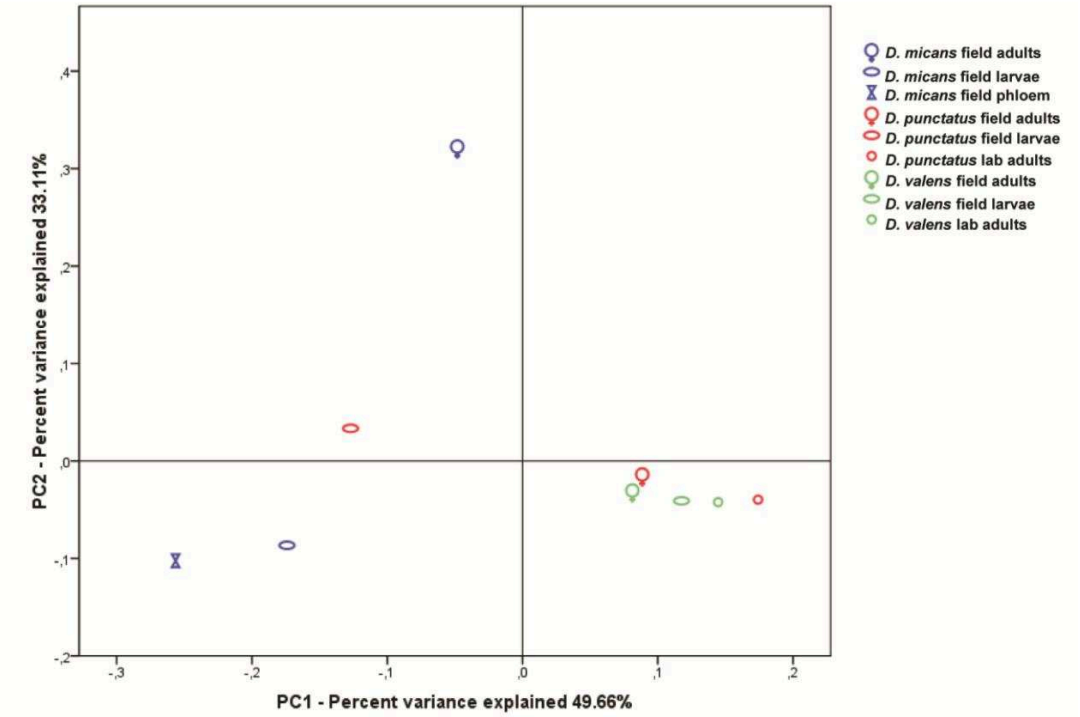

Fig. S2. Weighted Unifrac PCoA of the relative abundance of pyrosequenced bacterial OTUs in *D. micans*, *D. punctatus* and *D. valens* field and laboratory larvae, adults and distant phloem. There was no significant clustering of samples depending neither on origin (lab vs. field; ANOSIM: p=0.39) stages (field adults vs. larvae; p=0.39) and species (p=0.056).

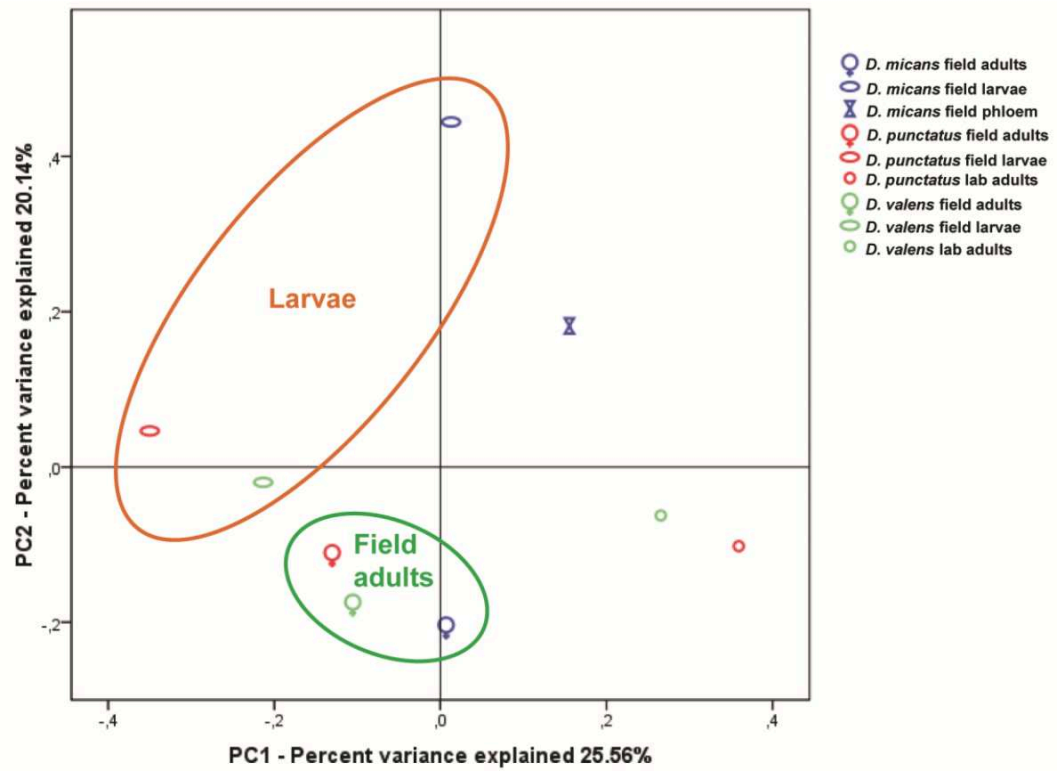

Fig. S3. Unweighted Unifrac PCoA of the relative abundance of pyrosequenced bacterial OTUs in *D. micans, D. punctatus* and *D. valens* field and laboratory larvae, adults and distant phloem. The ellipses denote significant differences between larvae and adults (ANOSIM $p = 0.023$).

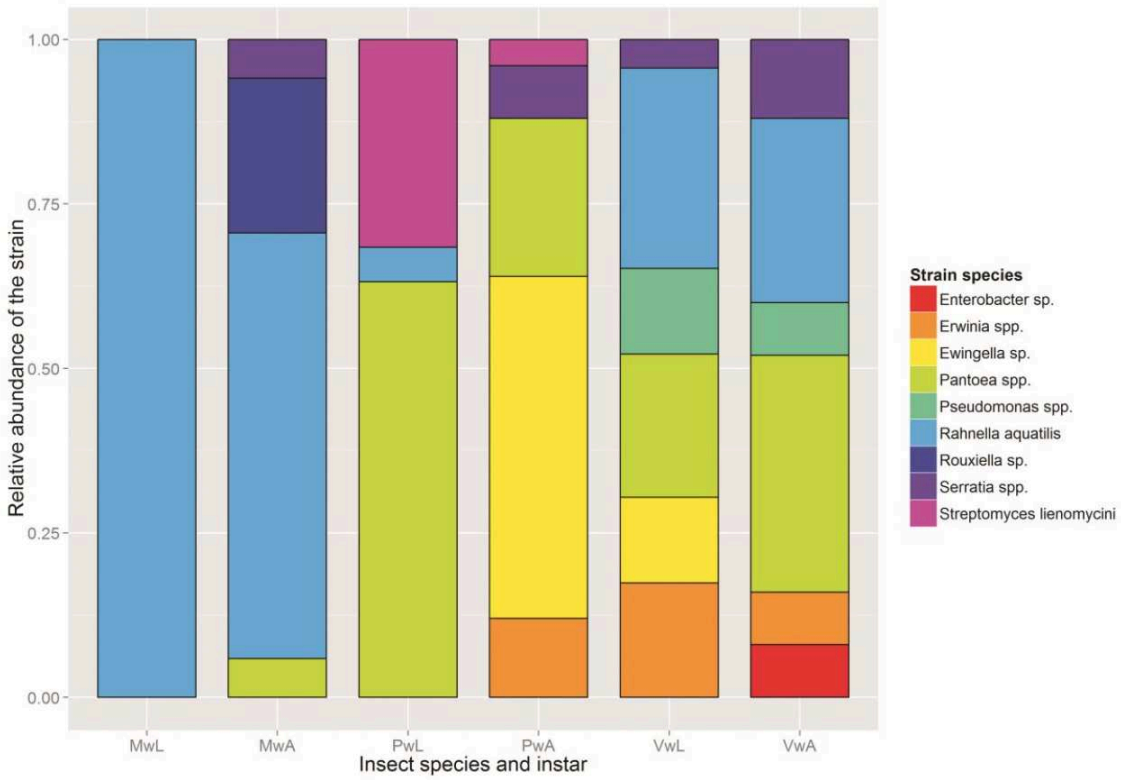

Fig. S4. Relative abundance of cultured bacterial taxa in *D. micans* (M), *D. punctatus* (P) and *D. valens* (V) field (w) larvae (L) and adults (A).

Fig. S5. Neighbor joining tree of cultured bacterial strains (yellow) and closest neighbors constructed using MOLE-BLAST for 16S sequences.

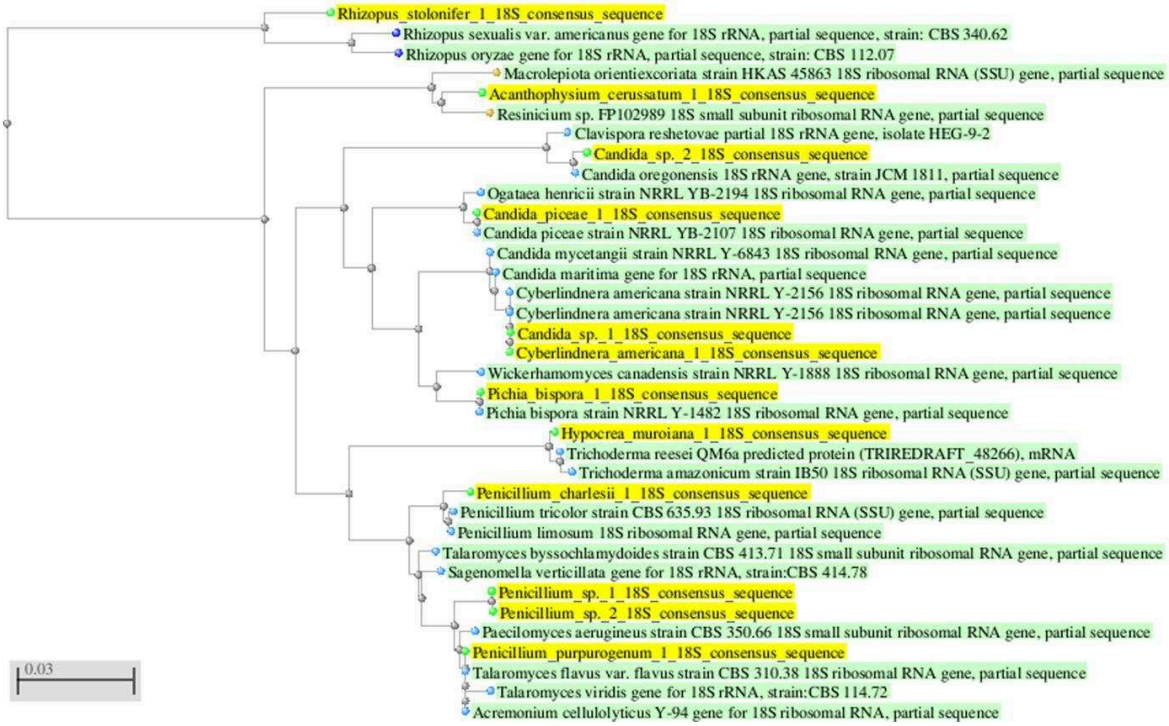

Fig. S6. Neighbor joining tree of cultured fungal strains (yellow) and closest neighbors constructed using MOLE-BLAST for 18S sequences.

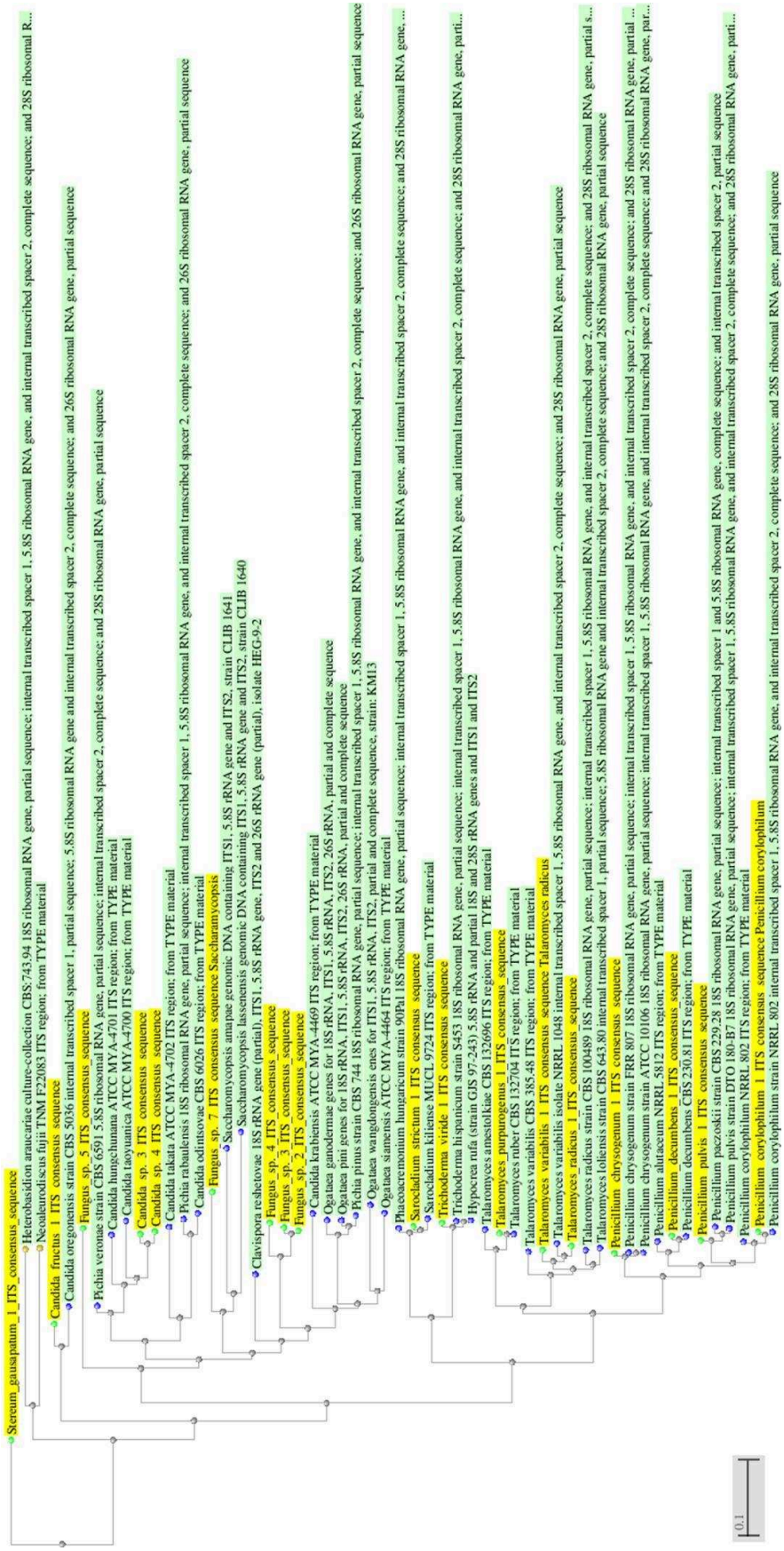

Fig. S7. Neighbor joining tree of cultured fungal strains (yellow) and closest neighbors constructed using MOLE-BLAST for ITS sequences.

4.11. REFERENCES

Delalibera, I., Vasanthakumar, A., Burwitz, B. J., Schloss, P. D., Klepzig, K. D., Handelsman, J., & Raffa, K. F. (2007). Composition of the bacterial community in the gut of the pine engraver, Ips pini (Say) (Coleoptera) colonizing red pine. *Symbiosis*, 43, 97–104.

Durand, A.-A., Bergeron, A., Constant, P., Buffet, J.-P., Déziel, E., & Guertin, C. (2015). Surveying the endomicrobiome and ectomicrobiome of bark beetles: The case of Dendroctonus simplex. *Scientific Reports*, 5(October 2015), 17190.

Hirsch, J., Strohmeier, S., Pfannkuchen, M., & Reineke, A. (2012). Assessment of bacterial endosymbiont diversity in Otiorhynchus spp. (Coleoptera: Curculionidae) larvae using a multitag 454 pyrosequencing approach. *BMC Microbiology*, 12(Suppl 1), S6.

Lu, F., Kang, X., Lorenz, G., Espino, L., Jiang, M., & Way, M. O. (2014). Culture-Independent Analysis of Bacterial Communities in the Gut of Rice Water Weevil (Coleoptera: Curculionidae). *Annals of the Entomological Society of America*, 107(3), 592–600.

Morales-Jiménez, J., Zúñiga, G., Ramírez-Saad, H. C., & Hernández-Rodríguez, C. (2012). Gut- associated bacteria throughout the life cycle of the bark beetle Dendroctonus rhizophagus Thomas and Bright (Curculionidae: Scolytinae) and their cellulolytic activities. *Microbial Ecology*, 64(1), 268–278.

Schloss, P. D., Delalibera, I., Handelsman, J., & Raffa, K. F. (2006). Bacteria Associated with the Guts of Two Wood-Boring Beetles: *Anoplophora glabripennis* and Saperda vestita (Cerambycidae). *Environmental Entomology*, 35(3), 625–629.

Scully, E. D., Geib, S. M., Carlson, J. E., Tien, M., McKenna, D., & Hoover, K. (2014). Functional genomics and microbiome profiling of the Asian longhorned beetle (*Anoplophora glabripennis*) reveal insights into the digestive physiology and nutritional ecology of wood feeding beetles. *BMC Genomics*, 15(1), 1096.

Tagliavia, M., Messina, E., Manachini, B., Cappello, S., & Quatrini, P. (2014). The gut microbiota of larvae of Rhynchophorus ferrugineus Oliver (Coleoptera: Curculionidae). *BMC Microbiology*, 14, 136.

Vasanthakumar, A., Handelsman, J., Schloss, P. D., Bauer, L. S., & Raffa, K. F. (2008). Gut Microbiota of an Invasive Subcortical Beetle, *Agrilus planipennis* Fairmaire, Across Various Life Stages. *Environmental Entomology*, 37(5), 1344–1353.

CHAPTER V

CONSERVED MICROBIOTA ACROSS CONIFER-FEEDING INSECTS MEDIATES THE DEGRADATION OF HOST PLANT DEFENSES IN THE PINE WEEVIL

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(In preparation)

5.1. ABSTRACT

The pine weevil (*Hylobius abietis*), a major pest of conifer forests throughout Europe, feeds on the trees' bark and cambium – tissues rich in terpenoids and toxic to many insect herbivores. Here, we report on the ability of the pine weevil's gut microbiota to degrade terpenoids *in vitro* and *in vivo*, findings that are consistent with our ability to annotate several genes of the diterpene degradation (*Dit*) gene cluster found in the metagenomic survey of the insect's bacterial community. Antibiotic treatment disrupts the core bacterial community of *H. abietis* and impairs the weevil's ability to digest terpenoids relative to individuals reared on their natural diet. Supplementing the diet of antibiotic-treated weevils with gut suspensions emanating from untreated insects restores the ability of insects to degrade terpenoids. Concordantly, insects reared on an artificial diet spiked with diterpenes were found to be more fecund than antibiotic-treated groups, or weevils reared on an artificial diet devoid of both terpenes and antibiotics, suggesting that microbe-driven catabolism of terpenes can enhance host fitness. Collectively, our findings are consistent with the hypothesis that the gut microbiota associated with the pine weevil, one that is conserved across other conifer-feeding insects, benefits *H. abietis* by mediating the degradation of plant defenses.

5.2. INTRODUCTION

The interactions between plants and insects are often mediated by secondary metabolites. In addition to acting as feeding attractants, these molecules can also be involved in plant defense (Mithoefer and Boland 2012). As defensive compounds, plant secondary metabolites are frequently deterrent or toxic to insects through the disruption of gut membranes which can impede digestion, hinder normal metabolism or ion and nutrient transport, among other effects (Mithoefer and Boland 2012). Herbivores have, in turn, evolved different mechanisms to overcome the noxious effects of plant defenses (Hammer and Bowers 2015). These involve changes in their feeding behavior, manipulation of plant defenses, as well as excretion, sequestration, and degradation of toxic chemicals (Després *et al.* 2007).

However, insects do not face these threats alone, but together with their gut microbes (Hammer and Bowers 2015), which can also be susceptible to plant toxins (Bakkali *et al.* 2008) and might even be their primary target (Mithoefer and Boland 2012). Symbiotic microorganisms have long been proposed as important players towards influencing interactions between plants and herbivores, e.g. by supplementing essential nutrients to insects or degrading complex dietary polymers and thereby making them accessible for the host's nutrition (Douglas 2009). Recently, these microbial functions have been expanded to encompass the manipulation (Chung *et al.* 2013) and degradation of plant secondary metabolites (Hammerbacher *et al.* 2013, De Fine Licht *et al.* 2013, Kohl *et al.* 2014, Ceja-Navarro *et al.* 2015, Welte *et al.* 2015).

Many herbivores exploit conifers as a food source. While these tissues are nutritionally imbalanced – due to their high C:N and C:P ratios, and low abundance of amino acids, vitamins and sterols – they are also enriched with chemical defenses (Keeling and Bohlmann 2006). In addition to phenolics, conifers produce a diverse mixture of resin acids, mainly composed of monoterpenes, and diterpene resin acids that, although constitutively expressed, can also be induced upon herbivory (Keeling and Bohlmann 2006). With more than 50,000 compounds, terpenes are the most diverse family of plant defenses described to date (Conolly and Hill 1991). Their noxious effects include antimicrobial properties (Rastogi *et al.* 1998, Lunde *et al.* 2000) as well as feeding deterrence and toxicity towards insects. Although the terpenoids' exact mode of action is still unknown in many cases (Gershenzon and Dudareva 2007), they seem to derive some of their toxic properties from the disruption of gut membranes due to their lipophilic nature or by causing neural damage through compromised ion channels (Keeling and Bohlmann 2006), and thus might be involved in conifer resistance against herbivores.

Although many insects are able to tolerate low terpene concentrations, higher amounts can act as deterrents or inhibitors (Zhao *et al.* 2011). Furthermore, a number of terpenes have been described to be toxic to conifer-feeding insects (Cook and Hain 1988, Raffa and Smalley 1995, Werner 1995). For example, white and Sitka spruce resistance against the white pine weevil (*Pissodes strobi*) is correlated to the content and inducibility of diterpenes (Tomlin *et al.* 2000) and monoterpenes (Harris 1983). Likewise, the Douglas fir pitch moth (*Synanthedon novaroensis*) is more successful attacking lodgepole pines containing low amounts of the monoterpene delta-3-carene (Rocchini *et al.* 2000). Similarly, the application of methyl jasmonate (MJ) on seedlings increases chemical defenses, including terpenes, in many conifer species (Martin *et al.* 2002, Heijari *et al.* 2005, Schmidt *et al.* 2205, Moreira *et al.* 2009), which correlates with higher resistance against the pine weevil (Heijari *et al.* 2005, Erbilgin *et al.* 2006, Sampedro *et al.* 2011).

Some environmental bacteria are known to degrade terpenes. For instance, *Pseudomonas abietaniphila* BKME-9 (Martin *et al.* 2000) and *Burkholderia xenovorans* (Smith *et al.* 2007) have been described to

degrade terpenes *in vitro.* However, the ability to detoxify and utilize terpenes is not limited to environmental microorganisms, but also occurs in symbiotic microbes associated with vertebrates and invertebrates. Goat rumen-associated bacteria can degrade several terpenes (Malecky *et al.* 2012), and some members of the gut community of bark beetles are capable of *in vitro* degradation of terpenoids (Boone *et al.* 2013, Xu *et al.* 2015). However, whether bacterial degradation of terpenes occurs *in vivo* as well has not yet been explored.

The pine weevil, *Hylobius abietis* (Coleptera: Curculionidae: Molytinae), feeds on bark and phloem of several conifer species. It is considered the most important conifer pest in managed forests in Europe (Leather *et al.* 1999, Nordlander *et al.* 2011) given its devastating impact on newly planted seedlings (Petersson and Orlander 2003). While adults feed both above and below ground, larvae complete their development underground tunneling in the bark of stump roots (Nordlander *et al.* 2005, Wallertz *et al.* 2006). Therefore, pine weevils encounter high concentrations of terpenoids throughout their life cycle. Despite the existing evidence of terpene noxious effects on herbivores, the pine weevil is able to cope with these compounds in low concentrations, although often high resin content deters them from feeding (Ericsson *et al.* 1988) in Scots pine. However, weevils feeding on Sitka spruce show a positive correlation between adult feeding, larval development and high concentrations of carbohydrates and resin (Langström and Day 2004), suggesting that the pine weevil is adapted to a wide range of terpene content. It is unknown whether the pine weevil has evolved mechanisms to overcome these compounds, and whether they do so on their own or through the association with symbiotic microorganisms.

Here we investigate whether the microbial associates of the pine weevil can play a role in overcoming conifer defenses. The gut microbiota of the pine weevil is geographically stable across Europe, especially within the most abundant bacterial family, the Enterobacteriaceae (Berasategui *et al.* 2016). The core microbiota of this insect consists of members of the genera *Erwinia*, *Rahnella,* and *Serratia*. This community assembly seems to be shaped, at least in part, by the nutritional resource these insects exploit (i.e. conifer bark and cambium). The same group of microbes is found, not only in the pine weevil, but also in other conifer feeding beetles, including several species of the genus *Dendroctonus* and *Ips pini* (Berasategui *et al.* 2016, Dohet *et al.* 2016, Adams *et al.* 2013, Hu *et al.* 2014, Cardoza *et al.* 2006) as well as wood-feeding wasps (Adams *et al.* 2011), but it is absent in weevils feeding on non-conifer food sources such as crops or ornamental plants (Berasategui *et al.* 2016). Among the different members of the community, species of the genera *Pseudomonas*, *Rahnella* and *Serratia* have been described based on metagenomics data (Adams *et al.* 2013) or phylogenetic inference (Berasategui *et al.* 2016) to contain many genes involved in diterpene degradation.

In this communication, we follow up on our broad survey of the gut community in the pine weevil to better understand why this consortium of microbes is enriched in bark feeding insects and whether they are involved in conferring resistance against plant terpenoid defenses encountered by the host. Towards this aim, we have performed *in vitro* chemical analyses to test whether the gut microbiota of *H. abietis* can degrade terpenes. We also highlight the terpene-degrading capabilities of this bacterial community *in vivo*, through bioassays aimed at manipulating the gut microbiota of *H. abietis*. Additionally, we have sequenced the bacterial metagenome of individuals feeding on different diets in order to explore the genetic underpinning of functional contributions of the microbes towards the host and whether the bacterial community contains diterpene degrading genes. Finally, we performed bioassays to assess the effect of both terpenes and gut microbes on the fitness of the insect host.

5.3. RESULTS

5.3.1. Terpene concentrations in feces of the pine weevil

We tested the hypothesis that terpenes are degraded within the weevil gut. Insects were fed on Norway spruce branches overnight and their feces were collected for the following 24 h. Total diterpene content in both food and feces was analyzed by gas chromatography-mass spectrometry (GC-MS). Our results show a reduction of 83% in diterpene content in feces relative to ingested material (Fig. 1), suggesting diterpene biodegradation during digestion.

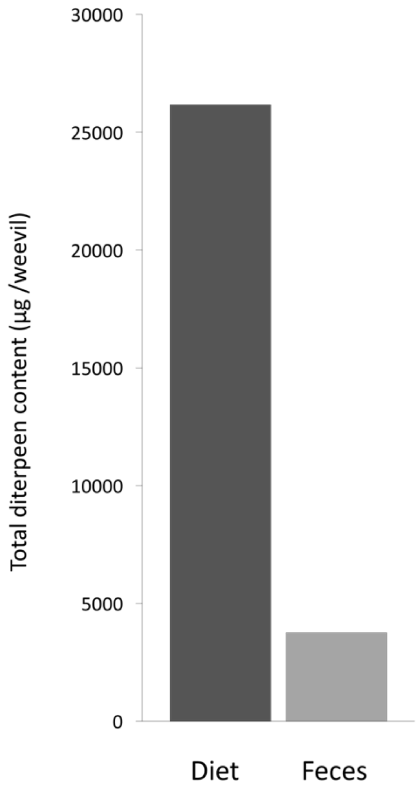

Figure 1. Amount of total diterpenes present in diet and pooled feces of weevils fed on Norway spruce. Total amount of diterpenes (µg) has been normalized by the number of weevils (n=62).

5.3.2. In vitro degradation of terpenoids by the weevil microbiota

To test whether the gut community of the pine weevil can degrade terpenes, we performed *in vitro* assays featuring gut suspensions from *H. abietis*. We performed the experiments with dehydroabietic acid because it is commercially available in high amounts. We prepared liquid LB media supplemented with dissolved dehydroabietic acid (DHAA) and inoculated vials with either overnight cultures of gut bacteria or sterile LB liquid media as controls. Using high pressure liquid chromatography, we analyzed the concentration of DHAA that remained in each vial every 24 hours. No significant differences in concentration were observed at the beginning of the experiment between treatments (repeated measures test, P=0.1). However, we observed a 20% reduction in the amount of DHAA in the presence of bacteria compared to controls within five days (repeated measures test, P=0.02) (Fig. 2).

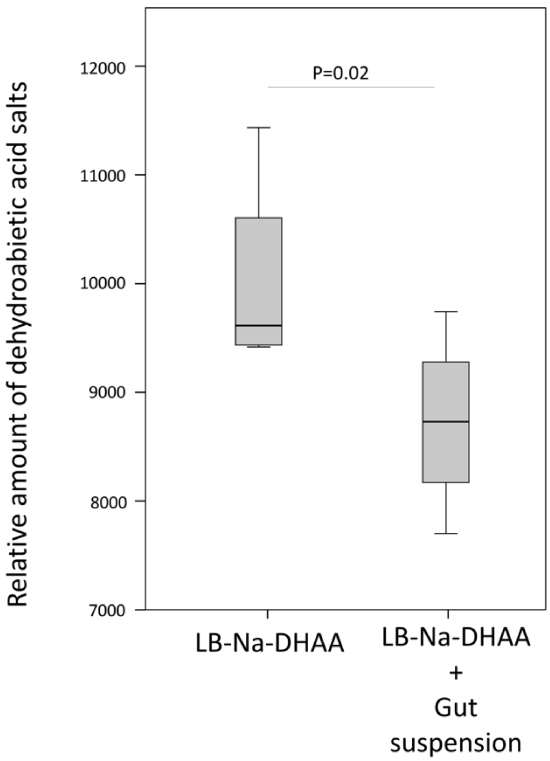

Figure 2. Relative amount of dehydroabietic acid in liquid cultures in the presence or absence of gut bacteria isolated from the pine weevil after five days of growth. Lines represent medians, boxes comprise the 25–75 percentiles, and whiskers denote the range. (ANOVA, P=0.02).

5.3.3. In vivo degradation of terpenoids

To test whether microorganisms mediate the degradation of terpenoids observed after passage through the pine weevil gut, we manipulated the gut microbes by adding the broad spectrum antibiotic rifampicin to a semi-artificial diet fed to the insect. Subsequently, we assessed the terpene content in the feces observing an increase in the amount of the volatile monoterpenes alpha- and beta-pinene, as well as the non-volatile diterpenes isopimaric and dehydroabietic acid, in the feces of antibiotic-treated individuals relative to the control group (ANOVA, P=0.05) (Fig. 3). Reinfection of the gut with the native community by supplementing a gut suspension of untreated individuals into the diet of antibiotic-treated insects restored the insect's biodegradative capacity, suggesting that gut microbes are responsible for the breakdown of such compounds within the host.

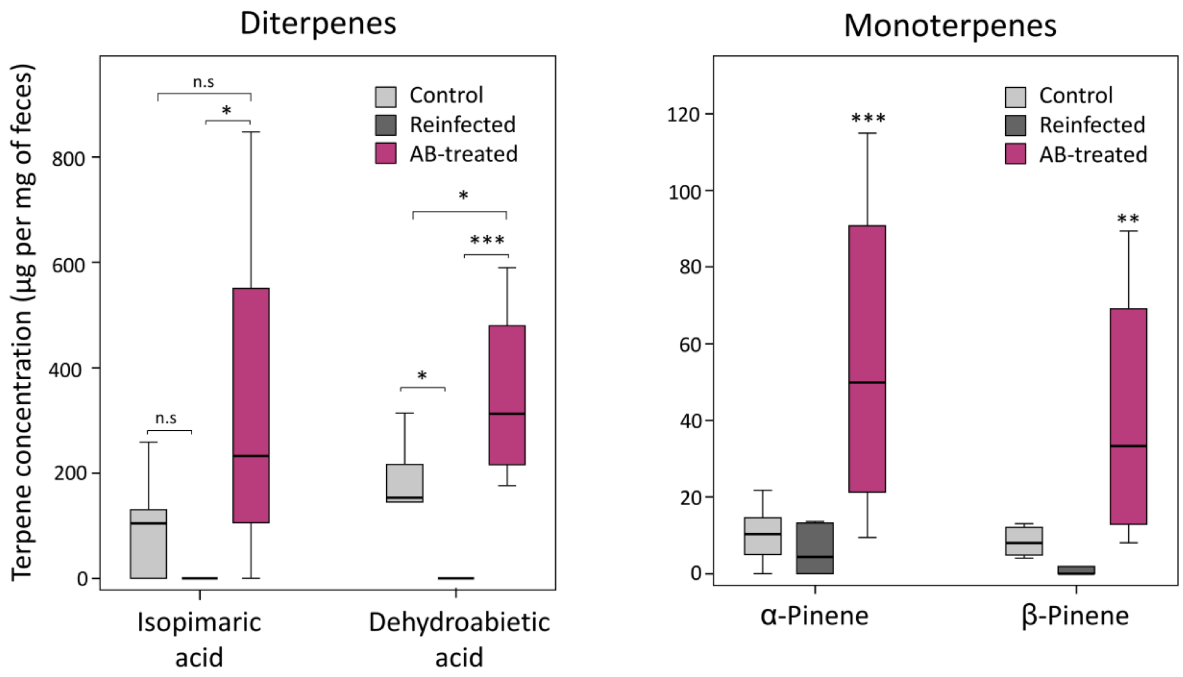

Figure 3. Concentration of the most abundant terpenoids in feces (ng/mg feces) of weevils feeding on different diets. Colori of boxes signifies the experimental treatment: Control, ground Norway spruce for 14 days; Reinfected, Norway spruce amended with a weevil gut suspension for 14 days; AB-reinfected, Norway spruce with antibiotics for 7 days and supplemented with a gut suspension for 7 days. Lines represent medians, boxes comprise the 25–75 percentiles, and whiskers denote the range. Abbreviations: n.s, not significant; *, $p<0.05$; **, $p<0.01$; ***; $p<0.001$.

5.3.4. Effect of terpenes and gut bacteria on host fitness

In order to assess the effect that terpenoids have on the pine weevil´s fitness we measured survival and fecundity of weevils feeding on different versions of an artificial diet. The first group of individuals feeding solely on artificial diet devoid of antibiotics and terpenes (AD) served as a control group. A second group was offered with an artificial diet supplemented with antibiotics (AD+AB). A third treatment included weevils feeding on artificial diet supplemented with terpenes (AD+T). Lastly, the fourth treatment comprised insects feeding on an artificial diet supplemented with both terpenes and antibiotics (AD+T+AB).

We observed no difference in survival rates depending on treatment (Mantel-Cox P=0.18; Breslow P=0.27; Tarone-Ware P=0.19) or sex (Mantel-Cox P=0.56; Breslow P=0.67; Tarone-Ware P=0.66) after 30 days (Fig. 4A). However, we observed differences between treatments in relation to the number of eggs laid (Fig. 4B). Individuals that fed on an artificial diet with terpenes and had their natural microbial community intact (AD+T) layed more eggs than individuals in any of the other three groups (P=0.05). Likewise, hatching rates of eggs laid by terpene-fed mothers with their native microbiota were significantly higher than in any of the three other groups (P=0.01, Fig. 4C).

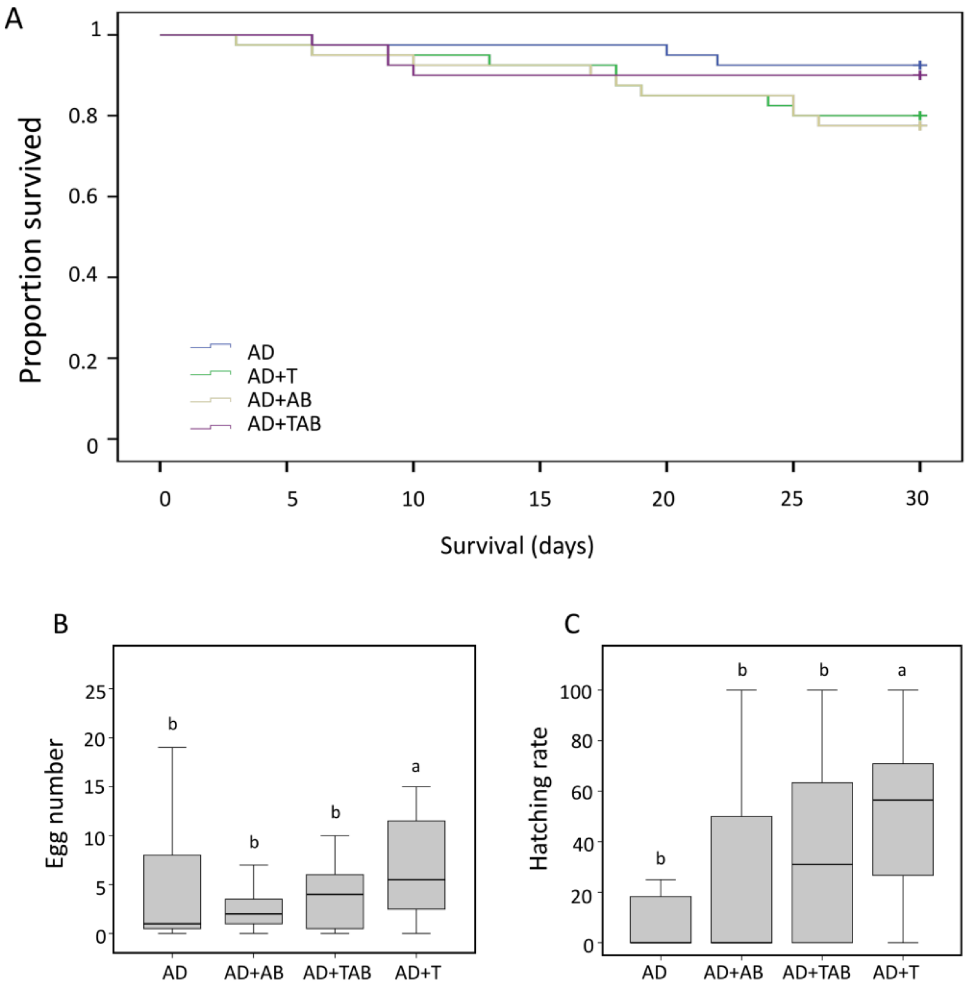

Figure 4. Fitness parameters of weevils feeding on different diets: AD, artificial diet (blue); AD+AB, artificial diet with antibiotics (yellow); AD+TAB, artificial diet amended with terpenes and antibiotics (purple); AD+T, artificial diet amended with terpenes only (green). In the box plots, lines represent medians, boxes comprise the 25–75 percentiles, and whiskers denote the range. (a) Survival: treatment (Mantel-Cox P=0.18; Breslow P=0.27; Tarone-Ware P=0.19); sex (Mantel-Cox P=0.56; Breslow P=0.67; Tarone-Ware P=0.66). (b) Number of eggs laid (ANOVA, P=0.05) and (c) egg hatching rate (ANOVA, P=0.01).

5.3.5. Metagenomic insights into symbiont-mediated terpene degradation

To identify the metabolic underpinnings of bacterial-mediated diterpene degradation in *H. abietis*, we sequenced the bacterial metagenome of beetles feeding on their natural food source (Norway spruce, *Picea abies*), an artificial diet (AD), as well as an artificial diet supplemented with antibiotics to disrupt the gut microbiota (AD+AB). Each library contained on average 15.3 million base pairs (Table S1), and assemblies resulted in an average of 49.983 contigs.

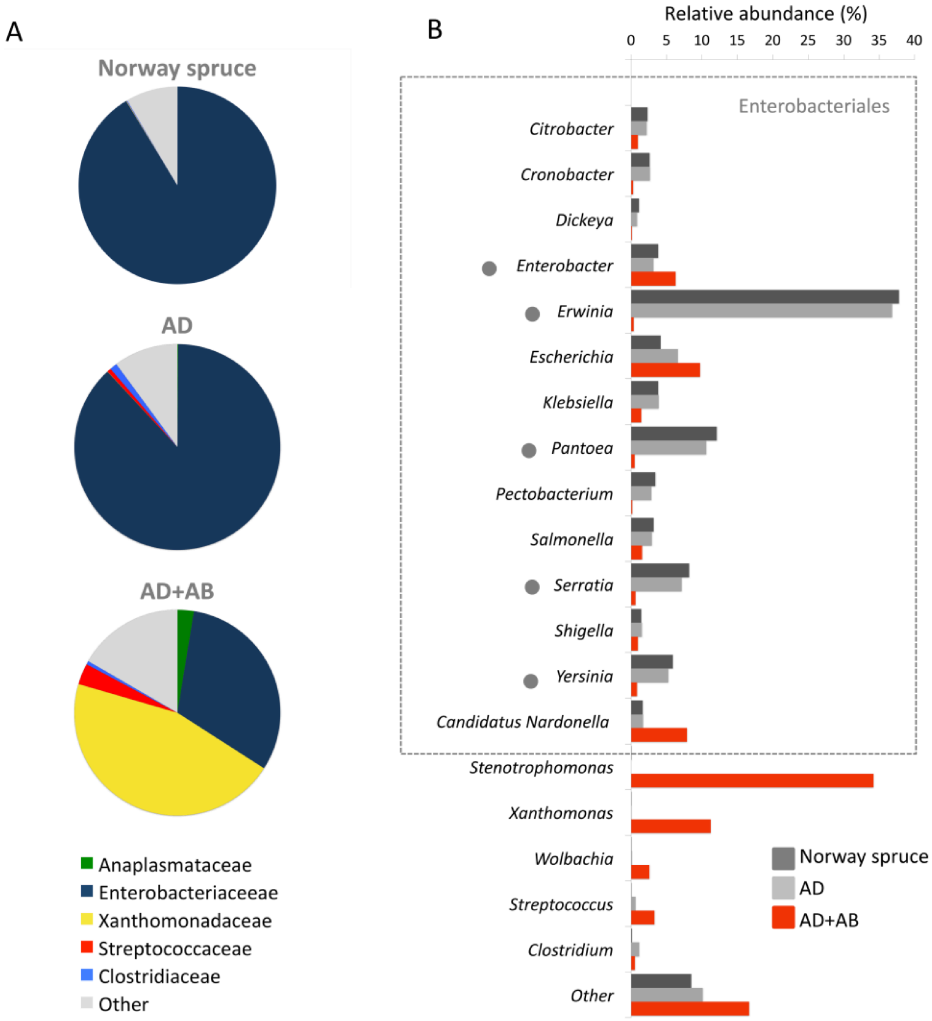

Figure 5. A) Taxonomic classification of the bacterial metagenome of weevils feeding on Norway spruce, artificial diet, and artificial diet with antibiotics, respectively. B) Abundance of different genera in the gut microbiota of weevils feeding on different diets (Norway spruce, artificial diet and artificial diet amended with antibiotics). Grey dots depict taxa previously found in the gut microbiota of the pine weevil (Berasategui *et al.* 2016). Dashed line clusters members of the Enterobacteriaceae family.

The metagenome assembly of pine-feeding weevils contained 35.370 contigs, out of which 72.1% were assigned to bacteria, whereas 27.5% were binned as belonging to eukaryotes, 0.3% to viruses, 0.03% were binned to archaea and 0.07% were not assigned to any group (Table S2). Consistent with our previous 16S rRNA-based survey of the insect's microbiome (Berasategui *et al.* 2016), community profiling using protein-coding genes revealed the gut microbiota of weevils feeding on a coniferous diet to be dominated by gammaproteobacterial associates that could be assigned to various Enterobacteriaceae genera (91.3%; Fig. 5A) including *Erwinia*, *Rahnella* and *Serratia* (Fig. 5B). Strikingly, all genera that appear in more than 1% abundance belong to the Enterobacteriaceae family, and nine out of fourteen had been previously reported to be present in the pine weevil's community (Berasategui *et al.* 2016).

In addition to the metagenome of spruce-feeding beetles, we also sequenced the bacterial metagenome of beetles reared on an artificial diet as well as beetles supplied with an artificial diet amended with antibiotics. Qualitatively, the gut bacterial community of weevils reared on the artificial diet remained very similar to the control treatment (Fig. 5A). The Enterobacteriaceae was the most abundant family (87.9%) followed by the Streptococcaceae and Clostridiaceae families (1.7% in total) (Fig. 5A). Additionally, all Enterobacteriaceae genera present in more than 1% abundance in spruce-fed weevils are present in artificial diet-fed insects, with the only addition of *Streptococcus sp.*

However, supplementing antibiotics into the artificial diet altered the microbiota, rendering it more diverse (Fig. 5A), with five families in abundances higher than 1%. In this group, the Xanthomonadaceae family is the most abundant (45.5%), followed by the Enterobacteriaceae (31.5%), Streptococcaceae (3.29%), Clostridiaceae (0.54%), Anaplasmaceae (specifically *Wolbachia*) (2.5%) and others (16.65%). Apparently, the Enterobacteriaceae family is the most susceptible to antibiotic treatment, resulting in a drastic reduction in their overall abundance compared to insects reared on their natural diet or an artificial diet devoid of antibiotics (Fig. 5B).

In order to explore the genetic correlates of symbiont-mediated diterpene degradation in the pine weevil, the three bacterial metagenomes sequenced in this study were screened for the presence of the *dit*-gene cluster. This cluster is predicted to be involved in the degradation of diterpenes as described in *Pseudomonas abietaniphila* BKME-9 (Martin *et al.* 2000, Smith *et al.* 2007, Smith *et al.* 2008). Genes encoding enzymes that had been previously described to degrade diterpenes were identified using BLASTn. The metagenome of beetles feeding on Norway spruce contained 10 out of 19 *dit* genes (Fig. 6). The same was true for beetles reared on the artificial diet, consistent with the minimal changes observed in the composition of the microbial community (Fig. 6). Phylogenetic binning of those sequences revealed that they predominantly belong to the Enterobacteriaceae family (Table S2), strongly suggesting that this is the main group involved in diterpene degradation. In addition to disrupting the compositional homeostasis of the bacterial associates in *H. abietis*, supplementing antibiotics into the artificial diet led to a near complete loss of *dit* genes (Fig. 6).

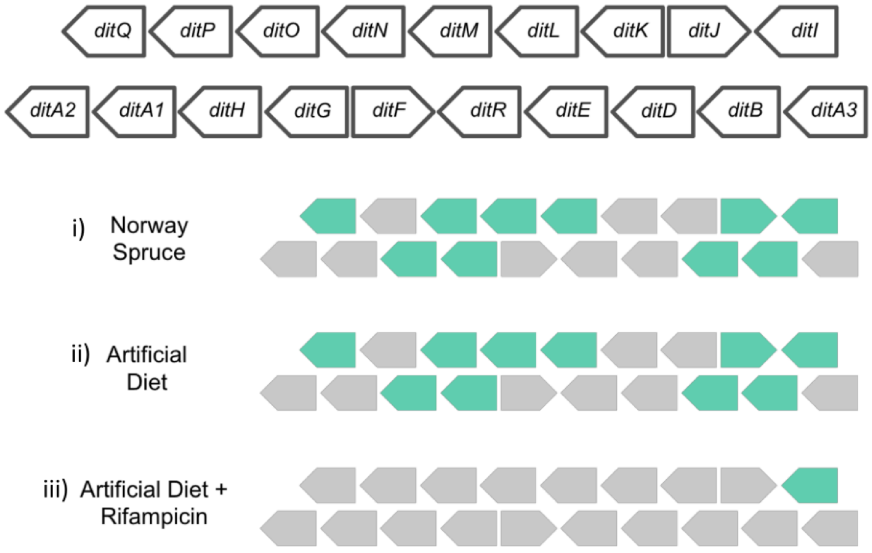

Figure 6. Diterpene gene cluster present (Martin *et al.* 2000) in the metagenome of weevils feeding on different diets: Norway spruce, artificial diet, and artificial diet amended with antibiotics. Coloring: turquoise, gene present; grey, gene absent.

5.4. DISCUSSION

Consistent with their involvement in many aspects of herbivore physiology, ecology and evolution, microbes have been shown to mediate plant-herbivore interactions in a number of insect groups (Tsuchida *et al.* 2004, Hosokawa *et al.* 2007). Microorganisms can aid in the exploitation of plant resources through the supplementation of essential nutrients, degradation of complex structural metabolites (Douglas 2009) and manipulating (Chung *et al.* 2013) or overcoming (Hammer and Bowers 2015) plant defenses. Microbial symbionts of herbivorous animals have long been suspected to aid in the detoxification of plant secondary metabolites and thereby contribute to host fitness, but direct experimental evidence has remained scarce (but see Kohl *et al.* 2014, Ceja-Navarro *et al.* 2015). Here we show that gut microbes of the pine weevil contain terpene degradation genes and are able to degrade mono- and di-terpenes *in vitro* and *in vivo*. Furthermore, in the presence of the native gut microbiota, terpene supplementation to the diet enhances the beetle's fecundity and egg hatching rate.

In agreement with our previous report using 16S rRNA profiling (Berasategui *et al.* 2016), taxonomic classification of the gut bacterial metagenome of the pine weevil revealed a community dominated by the Enterobacteriaceae family. Specifically, members of *Erwinia sp.*, *Pantoea sp.*, *Serratia sp.*, and *Yersinia sp.*, encompassed 90% of the whole microbiota. Although we detected *Wolbachia* in all three metagenomes sequenced, its abundance is markedly lower than previously reported using 16S rRNA data (Berasategui *et al.* 2016). Although differences in *Wolbachia* titers within a single insect population have been previously described (Müller *et al.* 2013), it is possible that differences in *Wolbachia* abundances arise from bias in the PCR required for 16S pyrosequencing, which is not required for metagenome sequencing. The very low abundance of Firmicutes in the present study is consistent with that of weevils collected in the same location (Spain) which were devoid of this bacterial family (Berasategui *et al.* 2016). This further supports the idea of the Enterobacteriaceae family being stable and conserved constituents of the gut microbiota in the pine weevil, with considerable variability affecting other members of the community. In any case, the conserved fraction of the microbiota among both pine weevil studies, and its overlap with that of other conifer-feeding beetles as well as a conifer feeding wasp (Adams *et al.* 2011) suggests that the bacterial community might be acquired from the environment and may be adaptively significant.

This community assembly remained unaltered after two weeks of beetle feeding on a completely artificial diet (Fig. 4), indicating that in addition to being conserved among the pine weevils and other conifer-feeding beetles (Berasategui *et al.* 2016), the community is also resilient to changes in diet. However, the addition of antibiotics did alter the community composition, reducing the relative abundance of most Enterobacteriales (with the exception of *Enterobacter* sp., *Escherichia* sp., and the bacteriome-localized endosymbionts), and increasing that of *Stenotrophomonas* sp., *Xanthomonas* sp., and *Wolbachia* (Figure 5B).

Our previous inference of metagenomic functions via PICRUSt (Berasategui *et al.* 2016) suggested the enrichment of a *dit*-gene cluster among *H. abietis*' bacterial associates, one potentially involved in diterpene degradation *in vivo*. Functional annotation of the bacterial metagenome in the present study revealed the presence of ten out of the 19 known *dit* genes in beetles feeding on their natural food source as well as on an artificial diet (Fig. 6). The supplementation of antibiotics to the artificial diet resulted in the loss of all but one *dit*-gene (Fig. 6).

Regarding the propensity for diterpene degradation using a seemingly incomplete *dit* cluster, studies have shown that not all 19 genes are required for bacterial catabolism of terpenoids (Martin and Mohn 2000, Smith *et al.* 2004, Smith *et al.* 2007). For example, knocking out ditR does not impair the growth of *P. abietaniphila* BKME-9 on diterpene-rich media (Martin and Mohn 2000). Conversely, knocking out ditQ,

restricts growth of *P. abietaniphila* on dehydroabietic acid but not on abietic acid (Smith *et al.* 2004). Additionally, ditI, ditH, ditF all reported as essential keystone genes for diterpene degradation – were found to be present in our metagenomic survey of *H. abietis*' gut bacterial community (Fig. 6). Parallel to previous findings (Adams *et al.* 2013), phylogenetic binning of *dit*-gene sequences annotated within our metagenomes revealed that most of the sequences belonged to taxa from the Enterobacteriaceae, strongly supporting the involvement of this bacterial family in the degradation of diterpenes within *H. abietis*' gut. Despite belonging to the Enterobacteriaceae, we can exclude the primary endosymbiont of weevils (*Nardonella* sp.) as a driver of this function, given that it is not affected by antibiotic treatment (Fig. 5B) and thus cannot account for the different occurrence of *dit*-genes we observe in the metagenomic survey, nor the reduction in terpene concentration seen in this study.

Our quantification of diterpenes in feces of the pine weevil relative to ingested pine tissue revealed an 80% decrease in total diterpene content (Fig.1). We tested the possibility of microbial degradation of terpenoids by performing *in vitro* assays in liquid media that contained dehydroabietic acid (DHAA) in solution. Our results show a 20% reduction in DHAA concentration within five days (Fig. 2). To confirm that the microbiota also degrades terpenoids inside the insect, we manipulated the gut community through the addition of antibiotics to the insect diet. Our results demonstrate that the degradation of both mono- and diterpenes decreased upon addition of antibiotics (Fig. 3). Moreover, degradation was restored in antibiotic-treated insects upon supplementing their native bacterial community through the diet. Both *in vitro* and *in vivo* assays highlight the importance and contribution of gut bacteria towards terpene degradation.

Free-living microbes isolated from pulp mill wastewater and forest soil, such as *Pseudomonas abietaniphila* BKME-9 and *Burkholderia xenovorans* LB400, are described to degrade diterpenes (Smith *et al.* 2008) and are able to utilize them as their sole carbon source (Martin *et al.* 2000, Morgan and Wyndhan 2002, Smith *et al.* 2007). Likewise, degradation of terpenes *in vitro* by symbiotic bacteria was previously described for close relatives of *Serratia*, *Rahnella* (both Enterobacteriales), *Pseudomonas*, and *Brevundimonas* isolated from the gut of bark beetles *(Dendroctonus ponderosae* and *D. valens*) (Boone *et al.* 2013, Xu *et al.* 2015). However, it is unclear how this affects the insect host's fitness and development.

Symbiotic degradation of plant secondary metabolites is not limited to terpenes, nor to insects. For instance, members of the gut community of the cabbage root fly (*Delia radicum*) harbor a plasmid (saxA) involved in degradation of their host plant's isothiocyanates (Welte *et al.* 2016). The coffee bean borer (*Hypotenemus hampei*) relies on *Pseudomonas fulva*, a member of its gut bacterial community, to completely degrade the alkaloid caffeine, thereby increasing the insect´s fitness (Ceja-Navarro *et al.* 2015). Likewise, desert woodrats (*Neotoma lepida*) harbor a gut microbial assembly that allow their hosts to exploit the toxic creosote bush (*Larrea tridentata*) through the degradation of phenolic compounds (Kohl *et al.* 2014, 2016). In addition to bacteria, symbiotic fungi can also degrade plant secondary metabolites for their hosts. *Lasioderma serricorne*, the cigarette beetle, harbors a symbiotic yeast (*Symbiotaphrina kochii*) in its digestive system that is able to degrade several plant toxins and use them as sole carbon sources (Dowd and Shen 1990). Similarly, the phenolics that leaf cutter ants (*Acromyrmex echinatior*) encounter in leaves, are detoxified by a laccase produced by the ant cultivated fungus (*Leucocoprinus gongylophorus*) (De Fine Licht *et al.* 2013).

To assess the impact of gut bacteria on the pine weevil's performance in the presence/absence of terpenes, we measured several fitness parameters on weevils feeding on different diets. We observed a fitness benefit for the weevil through higher fecundity and hatching rate when bacteria-harboring insects fed on terpene rich diet (Fig. 4B and 4C). Different hypotheses might explain how gut bacteria can enhance their host's fitness through the degradation of terpenes. It has been suggested (Wainhouse *et al.* 2001) that the diet´s nutritional and defensive features can cause differences in the pine weevil´s fecundity given that they affect the amount of resources that females can allocate for egg production. For

instance, microbial-mediated degradation of terpenes could result in breakdown products that the insect host can use as nutrients. However, this appears unlikely to have a major impact on host fitness for two reasons. First, unlike other plant defenses like glucosinolates in Brassica plants, coniferous terpenoids do not contain any nitrogen, which is one of the limiting nutrients in plant-based diets. Second, terpene-degrading bacteria could instead provide a nutritional benefit to their insect host through the supplementation of nitrogen, vitamins or sterols, all scarce resources in conifer bark and phloem. For example, Morales and colleagues (2009, 2012) demonstrated that members of the gut community of the conifer-feeding beetles *D. ponderosae*, (*Rahnella sp*. *Pantoea sp* and *Stenotrophomonas sp*.) and *D. rhyzophagous (Pseudomonas sp*, *Rahnella sp.* and *Klebsiella sp*.) can fix nitrogen. Likewise, the gut microbiota could provide a fitness benefit through a defensive role, such as providing resistance against parasites either through direct competition or through the synthesis of small molecules. Lastly, the degradation of terpenoids by symbiotic microorganisms could be a mechanism for terpene detoxification. Our bioassays show that organisms feeding on terpenes that lack microbes do not suffer higher mortality than those that have them, suggesting an intrinsic mechanism of insects to degrade terpenes. Often, insect counter-adaptations against plant defenses are not completely effective (Parr and Thurston 1972; Agrawal *et al.* 2012; Richards *et al.* 2012) and many insects combine several strategies to overcome them (Després *et al.* 2007, Hammer and Bowers 2015). In the case of the pine weevil, its gut microbiota might add to the intrinsic ability of the weevil to overcome terpenes and further benefit the insect through nutrient supplementation or protection against parasites.

While our previous work demonstrated that the pine weevil possesses a core microbiota belonging to the Enterobacteriaceae family that is conserved across different geographic locations and conifer-feeding beetle species (Berasategui *et al.* 2016), here we highlight that this microbial assembly can degrade its host plant chemical defenses and that the interaction between terpenes and the gut bacterial community is beneficial for host fitness. As such, we conclude that although the exact mechanism is still unclear, microbial degradation of terpenes mediates fitness benefits in the pine weevil. Given that (i) the taxonomic composition and the functional capabilities of this microbial assembly strongly resemble that of other bark beetles, wood-feeding wasps, and sawflies exploiting very similar ecological niches (Berasategui *et al.* 2016, Adams *et al.* 2011, Adams *et al.* 2013, Boone *et al.* 2013), and (ii) this assembly benefits fitness (at least in the pine weevil) in the presence of terpenes, the presence of microbes appears to represent a convergent adaptation to exploit coniferous resources.

5.5. MATERIAL AND METHODS

5.5.1. Insect collection and maintenance

Insects were collected in Neustadt, Lower Saxony (Germany) and in Galicia (Spain). German beetles were collected by leaving recently cut logs near a clear cut for some days and manually collecting the beetles that were attracted. Spanish beetles were collected with clean pitfall traps baited with α-pinene and ethanol (Nordlander 1987). Vials without lids were filled with ethanol, and the entrance blocked with bait-impregnated paper towels or cotton and placed leaning towards a pine branch. Once in the lab, the beetles were stored in darkness at 10°C in boxes of 50 individuals with moist paper, a container with soil and Norway spruce (*Picea abies*) twigs. Insects were brought to the lab bench one week before each experiment for acclimatization.

5.5.2. Semi-artificial and artificial diet preparation

Semi-artificial diet. Bark and cambium of Norway spruce branches were manually removed from the wood and frozen in liquid nitrogen. Needles were removed with a scalpel and the remaining tissues were homogenized manually with a mortar and pestle under liquid nitrogen. A 1.60 g portion of agar (Roth) was diluted in 50 mL of water and let cool to around 60°C. This mixture was added to 18.75 g of the homogenized spruce tissue and stirred until homogeneous. The antibiotic containing semi-artificial diet was prepared by adding the broad spectrum antibiotic rifampicin to the diet to a final concentration of 0.3% (w/v).

Complete artificial diet. This was prepared by modifying the diet used for seed-feeding pyrrhocorid bugs described in Salem *et al.* 2014 with the addition 6 mL of sunflower seed oil instead of 20 mL.

5.5.3. In vitro degradation of diterpenes

Six beetles were dissected under sterile conditions. Individual guts were suspended in 1 mL of PBS (phosphate-buffered saline). To separate bacteria from the gut walls, tissues were sonicated (50/60 Hz, 117 V, 1.0 Amp) for 30 s, macerated with a pestle and vortexed at medium speed for 10 s. Vials were centrifuged at 5,000 rpm to pellet host tissues and the supernatant was filtered using 10 µm syringe filters.

A 10 µL quantity of each bacterial suspension was inoculated in 10 mL LB media and grown overnight shaking at 220 rpm at room temperature. Overnight cultures were diluted to an optical density of 0.1 at 600 nm (OD_{600}) with LB media. To test whether the gut community of the pine weevil is able to degrade diterpenes, we inoculated 10 µL of the diluted bacterial culture in 990 µL LB media amended with dehydroabietic acid sodium salts (LB-NaDHHA). In the same way, 10 µL LB media was inoculated in control vials. The experiment was carried out in a 24-well flat bottom plate (Sarstedt) and was replicated six times. One plate was sampled for chemical analysis every day during five days. Upon sampling, the content of each well was transferred to Eppendorf tubes and centrifuged for 5 min at full speed to pellet bacterial cells. The supernatant was then transferred to glass vials for diterpene analysis. Chemical analyses were performed with an HPLC-UV.

NaDHAA salts were produced by dissolving dehydroabietic acid (Sigma, 1.33 g, 4.43 mmol) in 12 mL MeOH. This mixture was amended with an equimolar amount of $NaHCO_3$ (372 mg) dissolved in 3 mL of water. Since the reaction proceeded very slowly, the mixture was left standing in a closed vial for 14 days at room temperature. Subsequently, the solution was filtered with a syringe filter and evaporated to dryness using streaming nitrogen. The colorless residue of sodium dehydroabietate (NaDHABA) was checked for purity using NMR spectroscopy. Data revealed the sole presence of NaDHABA. Experimental determination of NaDHABA solubility gave a saturation concentration of 1.3 mg per ml of culture medium. In order to prepare growing media, 750 mg of this mixture was added to 1.5 L of LB media. After autoclaving (121°C, 20 min), the solution was vacuum-filtered to remove un-dissolved particles.

5.5.4. In vivo degradation of terpenoids

To assess the potential role of the gut bacteria in the degradation of terpenoids within the beetle gut, six weevils (three males and three females) in individual petri dishes were allocated to each of three treatments: (i) control, (ii) antibiotic, and (iii) reinfected. Control individuals were fed on control semi-artificial diet, whereas antibiotic-treated individuals were fed on semi-artificial diet amended with antibiotics. Reinfected individuals were fed on the antibiotic diet for half of the experiment and then switched to a semi-artificial diet amended with a gut suspension of untreated weevils for the remaining time. We generated this suspension by crushing the guts of four untreated weevils in 1 ml PBS and

vortexing. Insects were provided with 100 mg of diet every day that was supplemented with 10 µL of gut suspension in the case of the reinfected treatment or PBS in the control.

The experiment lasted for fourteen days, in which feces were collected daily and frozen at -20°C. Feces from the last day were extracted with 200 µl tert-butyl methyl ether (TBME) and shaken overnight. The solvent fraction of each sample was divided in half and transferred to new vials. One half was used for mono- and sesquiterpene analysis, while the other was derivatized with 20 µl of 0.2 M N-trimethylsulfonium hydroxide (TMSH) for subsequent diterpene analysis. Chemical analyses were performed with a GC-MS.

5.5.5. DNA extraction and Illumina-based metagenome sequencing

For one week, six weevils per group were reared on one of three different diets: (1) Norway spruce (*Picea abies*) twigs, (2) artificial diet (AD), or (3) artificial diet amended with antibiotics (AD+AB). Through dissection, the midgut region was harvested from every individual using sterile forceps and iris scissors. Once dissected, DNA was extracted from the midgut using the Microbiome Kit (Qiagen) following the manufacturer's instructions. Equimolar concentrations of samples from the same treatment were pooled, resulting in a single sample per treatment. Sequencing of a 150-bp library was conducted for each treatment using an Illumina Genome Analyzer IIx at the Genome Center of the Max Planck Institute in Cologne. The assembly was generated with Meta-Velvet v1.0.19 (Namiki *et al.* 2012) based on ~40 million quality-filtered read pairs and subjected to a gap-closing analysis. For annotation of gene content as well as taxonomic assignment, we used the Meta Genome Rapid Annotation using Subsystem Technology (MG-RAST) (Meyer *et al.* 2008).

We determined the community profile in the metagenomic dataset using the "Best Hit Classification" tool of MG-RAST to classify protein coding sequences (CDSs) on the basis of best BLASTP hits with a minimum identity cutoff of 60%, and a maximum e-value cutoff of 10^{-5}. Sequences were classified at the genus level (95% identity), and taxa with <1% abundance were removed. To examine the enrichment patterns of genes encoding diterpene degradation genes across the different treatments, we performed BLASTP against a custom data set of proteins belonging to the *dit* diterpene acid degradation pathway of *Pseudomonas abietaniphila* BKME-9, as previously described by Adams et al (2013).

5.5.6. Fitness assays

Male and female weevils, 80 of each, were randomly distributed in couples to plastic boxes. Each box contained one piece of moisture paper and an Eppendorf tube lid as diet container. Each box was randomly assigned to one of four treatments: (i) terpene artificial diet, (ii) antibiotic artificial diet, (iii) terpene-antibiotic artificial diet and (iv) artificial diet. The artificial diet was prepared as mentioned above. Beetles were subjected to the experiment for one month. As fitness parameters, survival, number of eggs laid and number of eggs hatched were recorded every 24 hours.

5.5.7. Statistics

***In vitro* assays**. Differences in the concentration of terpenes in liquid cultures were assessed in SPSS with a repeated measures ANOVA.

***In vivo* assays.** Differences in the amount of terpenes in the feces of weevils were analyzed in SPSS using a one-way ANOVA.

Fitness assays. The number of eggs laid and the hatching rate were analyzed in R with a generalized(?) linear model. Survival was analyzed in SPSS using three different statistical tests: Mantel-Cox, Breslow, and Tarone-Ware.

5.5.8. Chemical analyses

GC-MS analyses

62 weevils were starved for 4 days in plastic boxes and sprayed with water daily to maintain humidity. Following 4 days of starvation, insects were fed on Norway spruce twigs *ad libitum*. After 24 hours, insects were transferred to glass petri dishes and allowed to defecate. Feces were collected and suspended in 5 ml water. We added the suspended feces to tert-butyl-methyl ether (1:5 v/v) in a total of 25 ml and let them shake for 20 hours, after which 5 ml of $(NH_4)_2CO_3$ were added to the mixture and vortexed. The solvent fraction was transferred to a new vial and concentrated to a volume of 500 µl, out of which 300 µl were used to analyze diterpenes as described in (Schmidt *et al.* 2011). The aqueous fraction was evaporated in a heater at 80°C and dry weight was calculated.

High Pressure Liquid Chromatograph (HPLC) analyses

Diterpene salts were analyzed on an HPLC (1100 series equipment, Agilent Technologies), coupled to a photodiode array detector (Agilent Technologies). Metabolite separation was performed on a Nucleodur Sphinx RP column (250 x 4.6 mm, 5 µm particle size, Macherey-Nagel, Düren, Germany). The mobile phase consisted of 0.2% formic acid in water (A) and acetonitrile (B) with a flow of 1 ml per minute with the following gradient: start 20% B, 0-20 min 20-100% B, 20-23 min 100% B, 23.1-28 min 20% B. For quantification, peaks were integrated at 220 nm and an external standard curve with an authentic standard (Sigma) was created.

5.6. Conflict of interests

The authors declare no conflict of interests.

5.7. Author Contributions

AB carried out the microbiological and chemical lab work, performed the data and statistical analyses, and wrote the manuscript; HS carried out the molecular work and metagenome analysis; CP synthetized the diterpenoid salts; VMS aided in chemical lab work; AS provided reagents; AB, AS, MK, JG and conceived of the study. All authors gave critical comments on the manuscript and gave final approval for publication.

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5.9. REFERENCES

Adams AS, Aylward FO, Adams SM, Aukema BH, Currie CR, Suen G, Raffa KF. (2013) Mountain pine beetles colonizing historical and naive host trees are associated with a bacterial community highly enriched in genes contributing to terpene metabolismo. *Applied and Environmental Microbiology.* 79 3468-3475.

Bakkali F, Averbeck S, Averbeck D, Waomar M. (2008) Biological effects of essential oils: a review. *Food Chemistry and. Toxicology.* 46 446–75

Berasategui A, Axelsson K, Nordlander G, Schmidt A, Borg-Karlson A-K, Gershenzon J, Terenius O, Kaltenpoth M. (2016) The gut microbiota of the pine weevil is similar across Europe and resembles that of other conifer beetles. *Molecular Ecology.* 25 4014-4031.

Boone CK, Keefover-Ring K, Mapes AC, Adams AS, Bohlmann J, Raffa KF. (2013) Bacteria associated with a tree-killing insecto reduce concentrations of plant defense compounds. *Journal of Chemical Ecology.* 39 1003-1006.

Cardoza YJ, Klepzig KD, Raffa KF. (2006) Bacteria in oral secretions of an endophytic insect inhibit antagonistic fungi. *Ecological Entomology,* 31 636-645.

Ceja-Navarro JA, Vega FE, Karaoz U, Hao Z, Jenkins S, Lim HC, Kosina P, Infante F, Northen TR, Brodie EL. (2015) Gut microbiota mediate caffeine detoxification in the primary insect pest of coffee. *Nature. Communications.* 6 7618.

Conolly JD, Hill RA (1991) Dictionary of terpenoids. London: Chapman & Hall.

Chung SH, Rosa C, Scully ED, Pfeiffer M, Tooker JF, Hoover K, Luthe DS, Felton GW. (2013) Herbivore exploits orally secreted bacteria to suppress plant defenses. *Proceedings of the National Academy of Sciences of the United States of America,* 110 15728-15733.

Cook SP, Hain FP (1988) Toxicity of host monoterpenes to *Dendroctonus frontalis* and *Ips calligraphus* (Coleoptera: Scolytidae). *Journal of Entomological Sciences.* 23 287–292.

Després L, David J-P, Gallet C. (2007) The evolutionary ecology of insect resistance to plant chemicals. *Trends in Ecology and Evolution,* 22 298-307.

Dohet L, Grégoire JC, Berasategui A, Kaltenpoth M, Biederman P. (2016) Bacterial and fungal symbionts of parasitic *Dendroctonus* bark beetles *FEMS Microbiology and Ecology.*

Douglas AE. (2009) The microbial dimension in insect nutritional ecology. *Functional Ecology,* 23 38-47.

Erbilgin N, Krokene P, Christiansen E, Zeneli G, Gershenzon J (2006). Exogenous application of methyl jasmonate elicits defenses in Norway spruce (*Picea abies*) and reduces host colonization by the bark beetle *Ips typographus*. *Oecologia*, 148(3), 426-436).

Ericsson A, Gref R, Hellqvist C, Langström B (1988) Wound response of living bark of Scots pine and its influence on subsequent feeding by *Hylobius abietis*. In: *Mechanisms of woody plant defenses against insects*, WJ Mattson, J Levieux, C Bernard-Dagan (Eds.). Springer Verlag. New York, Berlin, Heidelberg.

Gershenzon J, Dudareva N (2007) The function of terpene natural products in the natural world. *Nature Chemical Biology,* 3 408-414.

Hammer TJ, Bowers MD (2015) Gut microbes may facilitate insect herbivory of chemically defended plants. *Oecologia* 179 1-14.

Harris LJ, Borden JH, Pierce HD Jr, Oehlschlager AC (1983) Cortical resin monoterpenes in Sitka spruce and resistance to the white pine weevil, *Pissodes strobi* (Coleoptera: Curculionidae). *Canadian Journal of Forest Research,* 13 350–352.

Heijari J, Nerg AM, Kainulainen P, Viiri H, Vuorinen M, Holopainen JK (2005) Application of methyl jasmonate reduces growth but increases chemical defence and resistance against *Hylobius abietis* in Scots pine seedlings. *Entomolgia Experimentalis et Applicata,* 115 117-124.

Hosokawa T, Kikuchi Y, Shimada M, Fukatsu T. (2007) Obligate symbiont involved in pest status of host insect. *Proceedings of the Royal Society B-Biological Sciences*, 274 1979–1984.

Hu X, Yu J, Wang C, Chen H (2014) Cellulolytic bacteria associated with the gut of *Dendroctonus armandi* larvae (Coleoptera: Curculionidae: Scolytinae). *Forests,* 5 455-465.

Keeling CI, Bohlman J (2006) Genes, enzymes and chemicals of terpenoid diversity in the constitutive and induced defence of conifers against insects and pathogens. *New Phytologist* 170 665-675.

Kohl KD, Weiss RB, Cox J, Dale C, Dearing MD (2014) Gut microbes of mammalian herbivores facilitate intake of plant toxins. *Ecology Lett*ers, 10 1238-46.

Kohl KD, Stengel A, Dearing D (2016) Inoculation of tannin-degrading bacteria into novel hosts increases performance on tannin-rich diets. *Environmental Microbiology,* 18 1720-1729.

Langström B, Day K (2004) Damage and control of weevil pests. In: Bark and wood boring insects in living trees in Europe, a synthesis. F Lieutier, Day KR, Battisti A, Gregoire JC, Evans HF (eds.) Springer Netherlands. pp 415-444.

Leal I, White EE, Sahota TS, Manville JF (1997) Differential expression of vitellogenin gene in the spruce terminal weevil feeding on resistant versus susceptible host trees. *Insect Biochemistry and Molecular Biology,* 27 569– 575.

Lunde CS, Kubo I (2000) Effect of polygodial on the mitochondrial ATPase of *Saccharomyces cerevisiae*. *Antimicrobial Agents and Chemotherapy*, 44 1943–1953.

Martin VJJ, Mohn WW (2000) Genetic investigation of the catabolic pathway for degradation of abietane diterpenoids by *Pseudomonas abietaniphila* BKME-9. *Journal of Bacteriology,* 182 (13):3784-3793.

Martin D, Tholl D, Gershenzon J, Bohlmann J (2002) Methyl jasmonate induces traumatic resin ducts, terpenoid resin biosynthesis, and terpenoid accumulation in developing xylem of Norway spruce stems. *Plant Physiology,* 129 1003–1018.

Mason JM, Couture JJ, Raffa KF (2014) Plant-associated bacteria degrade defense chemicals and reduce their adverse effects on an insect defoliator. *Oecologia,* 175 901-910.

Meyer F, Paarmann D, D'Souza M, Olson R, Glass EM, Kubal M, Paczian T, Rodriguez A, Stevens R, Wilke A Wilkening J, Edwards RA (2008) The metagenomics RAST server - a public resource for the automatic phylogenetic and functional analysis of metagenomes. *BMC Bioinformatics* , 9:386.

Mithoefer A, Boland W (2012) Plant defense against herbivores: chemical aspects. *Annual Review in Plant Biology*, 63 431-450.

Morales-Jiménez J, Zúñiga G, Villa-Tanaca L, Hernández-Rodríguez C (2009) Bacterial community and nitrogen fixation in the red turpentine beetle, *Dendroctonus valens* LeConte (Coleoptera: Curculionidae: Scolytinae). *Microbial Ecology,* 58 879-891.

Morales-Jiménez J, Zúñiga G, Ramírez-Saad HC, Hernández-Rodríguez C (2012) Gut-associated bacteria throughout the life cycle of the bark beetle *Dendroctonus rhizophagous* Thomas and Bright (Curculionidae: Scolytinae) and their cellulolytic activities. *Microbial Ecology,* 64 268-278.

Moreira X, Sampedro L, Zas R (2009) Defensive responses of *Pinus pinaster* seedlings to exogenous application of methyl-jasmonate: concentration effect and systemic response. *Environmental and Experimental Botany,* 67 94-100.

Müller MJ, Dörr NC, Deprá M, Schmitz HJ, Valiati VH, Valente VL. 2013 Reevaluating the infection status by the *Wolbachia* endosymbiont in *Drosophila* Neotropical species from the *willistoni* subgroup. *Infection, Genetics and Evolution,* 19 232-239.

Namiki T, Hachiya T, Tanaka H, Sakakibara Y (2012) MetaVelvet: An extension of Velvet assembler to de novo metagenome assembly from short sequence reads. *Nucleic Acids Research*, 40 e155.

Raffa KF, Smalley EB (1995) Interaction of pre-attack and induced monoterpene concentrations in host conifer defense against bark beetle-fungal complexes. *Oecologia* 102 285–295.

Rastogi N, Abaoul J, Goh KS, Devallois A, Philogene E, Burgeois P (1998) Antimycobacterial activity of chemically defined natural substances from the Caribbean flora in Guadeloupe. *FEMS Immunology and Medical Microbiology,* 20 267–273.

Rocchini LA, Lindgren BS, Bennett RG (2000) Effects of resin flow and monoterpene composition on susceptibility of lodgepole pine to attack by the Douglas-fir pitch moth, *Synanthedon novaroensis* (Lep., Sesiidae). *Journal of Applied Entomology,* 124 87–92.

Salem H, Bauer E, Strauss AS, Vogel H, Marz M, Kaltenpoth M (2014) Vitamin supplementation by gut symbionts ensures metabolic homeostasis in an insect host. *Proceedings of the Royal Society B- Biological Sciences,* 281: 20141838.

Sampedro L, Moreira X, Zas R (2011) Resistance and response of *Pinus pinaster* seedlings to *Hylobius abietis* after induction with methyl jasmonate. *Plant Ecol*ogy, 3 397-401.

Schmidt A, Zeneli G, Hietala A, Fossdal C, Krokene P, Christiansen E, Gershenzon, J (2005) Induced chemical defences in conifers: Biochemical and molecular approaches to studying their function. In J. Romeo (Ed.), Chemical Ecology and Phytochemistry in Forest Ecosystems (pp. 1-28). Amsterdam: Elsevier

Smith DJ, Park J, Tiedje J, Mohn WW (2007) A large gene cluster in *Burkholderia xenovorans* encoding abietane diterpenoid catabolism. *Journal of Bacteriology,* 189:17, 6195-6204.

Smith DJ, Patrauchan MA, Florizone C, Eltis LD, Mohn WW (2008) Distinct roles for two CYP226 family cytochormes P450 in abietane diterpenoid catabolism by *Burkholderia xenovorans* LB400. *Journal of Bacteriology*, 190 1575-1583.

Tomlin ES, Antonejevic E, Alfaro RI, Borden JH (2000) Changes in volatile terpene and diterpene resin acid composition of resistant and susceptible white spruce leaders exposed to simulated white pine weevil damage. *Tree Physiology,* 20 1087–1095.

Tsuchida T, Koga R, Fukatsu T (2004) Host plant specialization governed by facultative symbiont. *Science* 303 1989.

Wainhouse D, Ashburner R, Boswell R (2001) Reproductive development and maternal effects in the pine weevil *Hylobius abietis*. *Ecological Entomology,* 26 655-61.

Welte CU, de Graaf RM, van den Bosch TJM, Op den Camp HJ, van Dam NM, Jetten MSM (2016) Plasmids from the gut microbiome of cabbage root fly larvae encode SaxA that catalyzes the conversion of the plant toxin 2-phenylethyl isothiocyanate. *Environmental Microbiology,* 18 1379-90.

Werner RA (1995) Toxicity and repellency of 4-allylanisole and monoterpenes from white spruce and tamarack to the spruce beetle and eastern larch beetle (Coleoptera: Scolytidae). *Environmental Microbiology,* 24 372–379.

Xu LT, Lu M, Sun JH. (2015) Invasive bark beetle associated microbes degrade a host defensive monoterpene. *Insect Science,* 23 183-90.

Zhao T, Krokene P, Hu J, Christiansen E, Björklund N, Langström B, Solheim, H, Borg-Karlson AK (2011) Induced terpene accumulation in Norway spruce inhibits bark beetle colonization in a dose-dependent manner. *PLOS one,* 6 e26649.

5.10. SUPPLEMENTARY MATERIAL

Table S1. Metagenome sequencing statistics. AD: artificial diet; AD+AB: artificial diet amended with antibiotics.

	Pine	AD	AD+AB
Total bp	19,858,286	11,466,037	14,831,739
Contigs	70014	17483	62452
Mean Seq. Length (bp)	283±392	655±355	237±336
Predicted proteins	56532	19351	43578
Identified proteins	13804	7111	5
Identified functional categories	7267	3832	773

Table S2. Taxonomical classification of metagenomics reads (%). AD: artificial diet; AD+AB: artificial diet amended with antibiotics.

	Pine	AD	AD+AB
Bacteria	72.1	76.8	20
Eukaryota	27.5	21.7	79
Viruses	0.3	0.7	0.8
Archaea	0.03	0.03	0.02
Unassigned	0.07	0.62	0.18
Total sequences	35370	20184	9647

CHAPTER VI

POTENTIAL APPLICATIONS OF INSECT SYMBIONTS IN BIOTECHNOLOGY

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7.1. ABSTRACT

Symbiotic interactions between insects and microorganisms are widespread in nature and are often the source of ecological innovations. In addition to supplementing their host with essential nutrients, microbial symbionts can produce enzymes that help degrade their food source as well as small molecules that defend against pathogens, parasites, and predators. As such, the study of insect ecology and symbiosis represents an important source of chemical compounds and enzymes with potential biotechnological value. In addition, the knowledge on insect symbiosis can provide novel avenues for the control of agricultural pest insects and vectors of human diseases, through targeted manipulation of the symbionts or the host-symbiont associations. Here, we discuss different insect-microbe interactions that can be exploited for insect pest and human disease control, as well as in human medicine and industrial processes. Our aim is to raise awareness that insect symbionts can be interesting sources of biotechnological applications and that knowledge on insect ecology can guide targeted efforts to discover microorganisms of applied value.

7.2. INTRODUCTION

Insects engage in a remarkable array of symbiotic interactions with microorganisms, which range from parasitic to mutualistic relationships. Among mutualisms, most of the best described associations are based on nutritional or defensive services provided by the symbionts to their hosts. In defensive interactions, the microorganisms protect their host against pathogens, parasites, parasitoids, or predators, often through the production of antimicrobial compounds or toxins (Flórez *et al.* 2015), whereas in nutritional mutualisms, they provide nutrients such as amino acids and vitamins or digestive enzymes that aid in the degradation of fastidious dietary polymers or the detoxification of noxious secondary

metabolites (Douglas 2009). Mutualistic relationships have played a major role in the evolution of insects, allowing them to exploit ecological niches that would have otherwise remained inaccessible (Sudakaran *et al.* 2015).

From a biotechnological perspective, symbiotic microorganisms constitute promising and mostly untapped sources for potential applications in medicine, bioremediation, industrial processes, and agriculture. As with free-living microbes, the efficiency of metabolites and enzymes produced by symbionts has been optimized for over millions of years by natural selection. In contrast to their free-living counterparts, however, symbiotic products have been tested for their efficacy in a eukaryotic host, increasing the chances of successful applications by humans due to the reduced risk of harmful side effects. In general, the knowledge on host-microbe interactions can be exploited in two different ways for biotechnological use (Fig. 1): (1) by targeting or utilizing symbiotic interactions to control agricultural pests or vector-borne diseases or to improve the health of economical important insects such as honeybees and (2) by the application of symbiont-produced compounds such as small bioactive molecules or enzymes for pharmaceutical use or industrial processes.

There is an accumulating body of research and review articles that have touched on insect symbiosis as biotechnological resources (Douglas 2007; Chaves *et al.* 2009; Jurkevitch 2011; Crotti *et al.* 2012; Ramadhar *et al.* 2014). However, we believe that the present minireview finds its value in providing a comprehensive overview of contexts in which insect symbiosis research may yield biotechnologically exploitable results, thereby bridging the areas of symbiosis research and biotechnology and raising awareness that the knowledge on insect ecology and symbiosis allows to target particular systems that are promising sources of biotechnologically interesting symbionts.

7.3. IMPLICATIONS OF INSECT SYMBIOSIS FOR BIOLOGICAL CONTROL OF AGRICULTURAL PESTS

The obligate reliance of many insects on their microbial partners provides a potential target for the biological control of devastating agricultural pests. As such, numerous studies have examined the importance of the associated microorganisms to host fitness and feeding ecology in an effort to manipulate these partnerships and render insect pests more vulnerable to broad-scale measures of population control by targeting the bacterial symbionts.

Some of the best-studied animal-bacterial mutualisms feature insects specializing on economically important crops, including aphids, whiteflies, mealybugs, and stinkbugs, as well as many others. Numerous manipulations have been administered to hinder the development and survivorship of insect pests by targeting the bacterial partner, mainly through the application of antibiotics (Baumann 2005) and/or by disrupting the symbiont's transmission route to the next host generation (Salem *et al.* 2015). While effective in highly controlled conditions (Nogge 1976; Tsuchida *et al.* 2004; Hosokawa *et al.* 2007; Salem *et al.* 2013), the use of these techniques to target pests in agricultural fields is either unfeasible technically, economically, and/or ethically, considering the drawbacks associated with antibiotic resistance as a byproduct of the wide-scale application of these compounds. However, based on the increasing interest in the development and use of antimicrobial peptides (AMPs) as a tool to control bacterial populations, coupled with the genetic tractability of some agricultural crops to produce AMPs (Francois *et al.* 2002), wide-scale delivery of these compounds via heterologous expression in the host plant may represent a targeted, cost-effective approach that may have broad implications, both in scale

and implementation. It is important to note, however, that AMPs—like antibiotics—could potentially harm mutualistic bacteria of plants and beneficial insects, so the ecological implications of this approach must be carefully investigated.

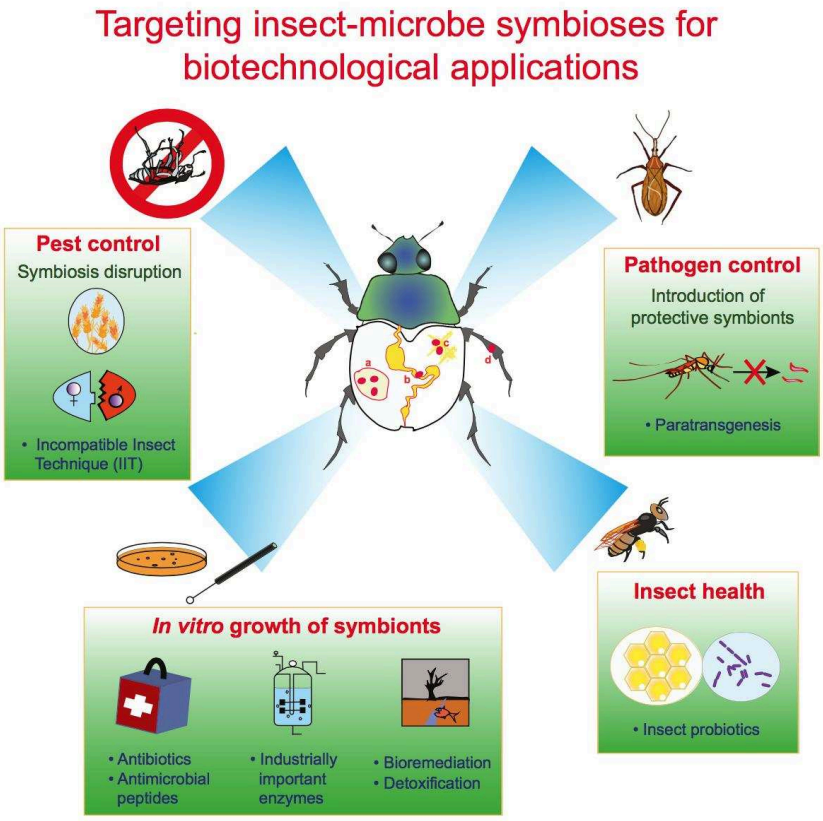

Fig.1 Biotechnological applications of targeting microbial symbionts in insects located (a) in specialized compartments (e.g., bacteriomes), (b) in the gut of the insect, (c) in insect tissues outside the gut (e.g., fat body), or (d) on the insect's cuticle. Targeting these symbiotic interactions can have broad applications in controlling populations of insect pests, increasing the survival of beneficial insects, and utilizing the symbionts for industrially important processes

Beyond demonstrations that the microbial associates of these agricultural pests influence survivorship and development, there is now growing evidence that the symbionts can also broaden the range of food plants that the insect host can utilize, which has profound implications for the economic risks that accompany a switch to an agricultural crop. While chromosomal loci of pea aphids (*Acyrthosiphon pisum*) have long been considered as predictors of host plant specialization, findings from Tsuchida and colleagues (2004) demonstrated that symbiotic bacteria can be similarly impactful, as evidenced by the ability of aphids to utilize white clover following specific infection by a facultative symbiont. Similarly, in whiteflies (*Bemisia tabaci*) specializing on sweet potatoes, a recent study has shown that infection by the endosymbiont Rickettsia results in insects that exhibit greater fecundity, faster development, higher survivorship, and increased production of females than observed in uninfected insects (Himler *et al.* 2011). Such benefits are thought to have contributed to the spread of the symbiont across whitefly populations at an unprecedented rate, thereby significantly impacting the ecology and invasive biology of its host. Along similar lines, the pest status of shieldbugs strongly correlates with the genotypic signature

of their bacterial partner (Hosokawa *et al.* 2007). Here, the legume-feeding shieldbug *Megacopta cribraria* suffered low survivorship and reproductive success when provisioned with soybeans as a sole food source; such effects were reversed when the insect's obligate symbionts were exchanged with symbiotic strains originating from the soybean-specializing shieldbug *Megacopta punctatissima* (Hosokawa *et al.* 2007). Consistent with this finding, an inva-sive population of *M. cribraria* in North America, which is utilizing soybeans, has a symbiont population with an overall nucleotide and functional profile resembling that of the Asian pest-conferring symbionts in *M. punctatissima* (Brown *et al.* 2014).

Classical methods of biological control of agricultural pests take advantage of parasitoids to reduce the insect pest population. In this context, knowledge on insect symbiosis could be of applied value, as some *Wolbachia* strains harbored by parasitoid insects induce thelytokous parthenogenesis in their hosts (Arakaki *et al.* 2000). Since only female parasitoids kill their hosts, a parthenogenetic phenotype in a parasitoid would have several advantages on insect pest control over the sexual one as noted by Stouthamer (1993) and Bourtzis (2008): (i) a drop in the cost of mass-producing parasitoids for release due to the fact that no males are produced, (ii) rapid population growth due to the higher number of females, and (iii) easier establishment because no mating is required.

7.4. SYMBIOSIS AS A TOOL TO LIMIT VECTOR-BORNE DISEASES

The use of microbial symbionts to limit the prevalence and competence of insect vectors of human diseases has been heralded as a promising research area to control the incidence of numerous devastating diseases, including malaria, dengue, yellow fever, and Chagas. Currently, two of the most active research areas include (1) the genetic transformation of bacterial symbionts to express molecules targeting the disease agent in the insect vector (paratransgenesis) and (2) manipulating the vector through the utilization of microbes that shorten life span and lower fertility of the insect host or that reduce its susceptibility to pathogens or parasites.

For the former, the most extensively developed model involves targeting the insect vector (*Rhodnius prolixus*) of the Chagas-causing protozoan *Trypanosoma cruzi* through the manipulation of the insect gut flora (Ben Beard *et al.* 2002). Early application of paratransgenesis in this system focused on the ability to genetically transform the gut symbiont *Rhodococcus rhodnii*, which co-localizes in the midgut with *T. cruzi*, in order to produce anti-trypanosomal effector molecules that target the parasite in the insect's gut (Durvasula *et al.* 1997). The transmission ecology of *R. rhodnii* (extracellular through coprophagy) and its amenability for *in vitro* cultivation and genetic transformation has presented the system as a useful platform to apply paratransgenesis as a way to limit the transmission of *T. cruzi*, as has been shown in semi-field trials (Durvasula *et al.* 1999). Additionally, Taracena and colleagues (2015) successfully introduced *Escherichia coli* expressing dsRNA for *Rhodnius* heme-binding protein and catalase into the gut of *R. prolixus*, which had serious fitness consequences for the bug by inducing systemic RNAi. Likewise, paratransgenesis has been used in tsetse flies to engineer *Sodalis glossinidius* to release anti-trypanosome nanobodies (antigen-binding molecules) in the host gut (De Vooght *et al.* 2014). A significant problem for paratransgenetic control of pest insects or disease vectors remains the delivery of manipulated bacteria to the insect under field conditions. However, an important step into this direction was the recent establishment of a targeted delivery system for genetically engineered bacteria using microencapsulation to control the spread of Pierce's disease by glassy-winged sharpshooters (*Homalodisca vitripennis*) under simulated field conditions (Arora *et al.* 2015).

In an effort to inhibit the transmission of mosquito-borne filariasis, *Wolbachia* strain wMelPop was successfully used to provoke a sustained insect immune response that incurs a heavy metabolic burden on the insect host (Kambris *et al.* 2009). This results in a shorter life span of infected mosquitoes, which reduces the possibility of disease transmission, since the parasite requires a long incubation time relative to the average life span of an individual mosquito. The upregulation of the immune system was also found to eliminate lymphatic filariasis from the vector, thereby disrupting its transmission (Kambris *et al.* 2009). *Wolbachia* wMelPop has also been successfully utilized to reduce the life span of laboratory cultures of the mosquito vector of dengue (*Aedes aegypti*), thereby compromising the ability of the virus to establish in the insect and effectively blocking subsequent transmission to a mammalian host (McMeniman *et al.* 2009). Most promising, however, is the strong inhibitory effect of *Wolbachia* wMel strain on dengue establishment within mosquitoes. Lab and field examination of *Wolbachia* effects on the epidemiology of dengue revealed that the bacterial symbiont is able to approach fixation in mosquito populations within a few generations (Hoffmann *et al.* 2011). In addition to dengue, other human pathogens (e.g., Chikungunya, Plasmodium) are markedly reduced (1500-fold) in mosquitoes infected with *Wolbachia* compared to untreated individuals (Moreira *et al.* 2009; Walker *et al.* 2011), thereby providing an excellent example for how symbioses between insects and microbes could be harnessed as a means for the biological control of vector borne diseases.

7.5. INCOMPATIBLE INSECT TECHNIQUE

Another potential *Wolbachia*-based approach to reduce both insect pests and vector-borne diseases is the incompatible insect technique (IIT). This procedure is analogous to the sterile insect technique (SIT) (Knipling 1955). Both methods rely on the mass release of sexually active but incompatible males into the wild that will mate with virgin females resulting in non-viable eggs (Laven 1967; Zabalou *et al.* 2004). SIT relies on various methods to achieve sterility in males, irradiation being the most common one. By contrast, IIT takes advantage of *Wolbachia*-induced cytoplasmic incompatibility (CI) or any other symbiont-induced reproductive incompatibility. *Wolbachia*-induced CI results in the death of embryos resulting from matings between *Wolbachia*-infected males and uninfected females (unidirectional CI) as well as from those involving individuals that are infected with different *Wolbachia* strains (bidirectional CI) (Werren 1997). In both examples, the release of infected males leads to a lower female fertility and can ultimately lead to the suppression of the population given enough time and constant release of incompatible males (Bourtzis *et al.* 2014).

The first successful application of IIT took place as early as in 1967 in Myanmar, where a population of the lymphatic filarial vector, the mosquito *Culex pipiens*, was eradicated, although the reasons behind the sterility were unknown at the time (Laven 1967). IIT has also been successfully tested against agricultural pests. Naturally, *Wolbachia*-free Mediterranean fruit flies (*Ceratitis capitata*) have been transinfected with a *Wolbachia* strain from the closely related cherry fruit fly *Rhagoletis cerasi* (Zabalou *et al.* 2004). This transinfection caused both unidirectional as well as bidirectional CI, opening the possibility for using it as an environmentally friendly pest control strategy. Equally promising are the results from Atyame and colleagues (2011). The *Wolbachia* strain wPip (ls) from *C. pipiens* was introgressed into *Culex quinquefasciatus* from four different islands of the South-Western Indian Ocean. In addition to 100 % embryo lethality from matings between sterile males and all tested field females, most crosses between introgressed females and field males were incompatible (Atyame *et al.* 2011).

However, the accidental release of *Wolbachia*-infected females could reduce the efficiency of the IIT by leading to the establishment of a viable *Wolbachia*-infected population as well as increase the risk of disease transmission since female insects are the transmitting vector. Therefore, different biological, genetic, and transgenic approaches to eliminate females early in the process by separating them from males have been developed (Laven 1967; Sweeny and Barr 1978; Condon *et al.* 2007; Brelsfoard *et al.* 2009). In addition to these, IIT itself could prevent the establishment of a transinfected population. The release of two reciprocally incompatible lines would result in most of the matings being incompatible (Bourtzis and Robinson 2006), thus reducing the number of potentially infectious females. An additional solution, and potentially the most promising, is to couple IIT with radiation-SIT (Bourtzis *et al.* 2014) since, for instance, tephritid flies as well as *Aedes polynesiensis* females can be sterilized with lower radiation than males (Bakri *et al.* 2005; Brelsfoard *et al.* 2009).

7.6. INSECT SYMBIONTS AS PROBIOTICS

Several studies have shown that sterile mass-reared Mediterranean fruit flies subjected to SIT are less successful than wild males at competing for wild females (Juan-Blasco *et al.* 2013). In addition to this, irradiation of males for SIT also results in an altered gut microbiota as compared to non-irradiated males. Ben Ami and colleagues (2010) showed that supplementation of the diet with *Klebsiella oxytoca* as probiotics (one of the most abundant taxa in fruit flies Vienna 8 strain and in wild fruit flies) rescues male competitiveness by shortening their mating latency. Likewise, the addition of *Enterobacter* sp. to larval diet results in higher pupal and adult recovery as well as shorter developmental time in all life stages of male fruit flies (Augustinos *et al.* 2015). Therefore, further examination of insect symbionts as probiotics could be valuable in the efforts to develop more successful SIT applications.

Similarly, the use of probiotics can be a valuable tool in protecting honey bee (*Apis mellifera*, Hymenoptera) populations, which are declining worldwide, probably due to a combination of pesticide use by humans and infection by parasites and pathogens (Cornman *et al.* 2012). For instance, the bacterium *Paenibacillus larvae* is responsible for the American foulbrood disease (AFB) within the insect's gut, killing the larvae before pupation. Lactic acid bacteria (LAB) of the genera *Lactobacillus* and *Bifidobacterium* have recently been isolated from the honey stomach (Olofsson and Vásquez 2008) and are potential probiotic candidates for enhancing honey bee immunity. In vitro and in vivo studies indicate that LAB show total inhibition of *P. larvae* in agar diffusion assays and addition of LAB to the larval diet significantly reduced AFB infection (Forsgren *et al.* 2010). This effect may be mediated by a direct inhibition of pathogen proliferation or through stimulation of the host's immune system (Evans and Lopez 2004). Other members of the honey bee gut community such as *Enterococcus* (Carina Audisio *et al.* 2011) and *Actinomycetes* (Promnuan *et al.* 2009) also produce antimicrobial compounds that have potential application in maintaining honey bee colony hygiene, as well as in preventing gut infection. Beneficial bacteria may thus provide interesting avenues for enhancing health and fitness of agriculturally important insects such as pollinators.

7.7. SYMBIONT-PRODUCED COMPOUNDS WITH ANTIMICROBIAL ACTIVITY

Antimicrobial secondary metabolites find important applications in human medicine and agriculture. However, the increasing resistance of human pathogens and the reduced discovery rate of novel compounds pose significant problems that threaten to result in the reappearance of human diseases that were thought to be defeated. In this context, insect-associated microbes present promising sources of novel bioactive compounds that are only beginning to be discovered and exploited (Dettner 2011). In particular, defensive insect-bacteria symbioses are interesting targets for natural products discovery, as the involved secondary metabolites have been tested over millions of years by natural selection for their efficacy against antagonists as well as for the lack of harmful side effects on the eukaryotic host (Flórez *et al.* 2015). In general, symbiont-produced defensive compounds are employed by insects in two different contexts: (i) as a protection of the host or its offspring against antagonistic micro- or macroorganisms or (ii) as weed killers in insect fungiculture (Kaltenpoth 2009; Ramadhar *et al.* 2014).

Microbial symbionts providing chemical defense to the host against predators, parasites, parasitoids, and pathogens occur in several insect taxa, including beetles, psyllids, planthoppers, and solitary wasps. In staphylinid beetles of the genus *Paederus*, symbiotic *Pseudomonas* bacteria produce the polyketide pederin that deters predatory wolf spiders (Kellner 2001; Kellner 2002; Piel 2002). A similar compound called diaphorin has recently been found to be produced by an intracellular symbiont of psyllids and suspected to confer protection against as yet unknown predators (Nakabachi *et al.* 2013). In another hemipteran insect, the brown planthopper *Nilaparvata lugens* (Delphacidae), an *Enterobacter* symbiont produces the antimicrobial compound andrimid with activity against pathogens of the planthopper's host plant (Fredenhagen *et al.* 1987). Finally, two different insect taxa—a group of solitary wasps and a weevil—employ symbiont-produced antimicrobials for protection of their developing offspring against mold fungi. The Candidatus *Streptomyces philanthi* symbionts of solitary beewolf wasps in the genera *Philanthus*, *Trachypus*, and *Philanthinus* produce a mixture of streptochlorin and at least eight different piericidins that defend the larva inside the cocoon against opportunistic mold fungi (Kaltenpoth *et al.* 2005; Kroiss *et al.* 2010; Kaltenpoth *et al.* 2014). And the leaf-rolling weevil *Euops chinensis* teams up with the fungus *Penicillium herquei* that produces (+)-scleroderolide and thereby protects the larval cradle against microbial antagonists (Wang *et al.* 2015).

Analogous to human agriculture, the domestication of fungal cultivars for food has evolved independently in several insect lineages. Due to the necessity for protecting the fungus monoculture from pathogens, these systems are particularly promising potential sources for bioactive metabolites produced by the insect themselves or associated symbionts (Ramadhar *et al.* 2014). Concordantly, actinobacterial symbionts of different fungus-growing ant species have been found to produce a range of secondary metabolites with general antimicrobial activity or targeting specific fungal antagonists. These compounds include dentigerumycin (Oh *et al.* 2009a), pseudonocardones A–C, 6-deoxy-8-O-methylrabelomycin, and X-14881 E (Carr *et al.* 2012a), nystatin P1 (Barke *et al.* 2010), candicidin (Haeder *et al.* 2009), as well as actinomycins, antimycins, and valinomycins (Schoenian *et al.* 2011). Furthermore, a *Streptomyces* strain associated with the fungus-growing bark beetle *Dendroctonus frontalis* produces the antifungal compound mycangimycin (Oh *et al.* 2009b). Finally, microtermolides A and B were isolated from a termite-associated *Streptomyces*, but showed no bioactivity against the tested bacterial and fungal strains (Carr *et al.* 2012b). Although to our knowledge, none of the compounds involved in these symbiotic associations has so far been exploited for clinical application, several substances show interesting antimicrobial, antiparasitic, or anti-cancer activities and may therefore be of interest for human medicine. Future studies on insects with life histories that entail particularly high exposure to pathogens (e.g., fungicultural systems, insects that mass-provision their offspring and/or develop within the soil) will

likely uncover additional defensive symbioses and bioactive natural products of potential value for human application.

7.8. INSECT SYMBIONTS AS A SOURCE OF DIGESTIVE ENZYMES

Insects show remarkable adaptations to the exploitation of diverse nutritional resources, owing to the wide diversity of digestive enzymes produced by the insects themselves as well as the metabolic capabilities of symbiotic microorganisms that overcome the host's nutritional limitations. In addition to the supplementation of essential nutrients such as amino acids, vitamins, and sterols, gut symbionts can also provide beneficial digestive enzymes when the host's diet is specialized, contains refractory substrates, and is deficient in nutrients and/or when insects colonize novel niches for which their own metabolic repertoire is inadequate. The following sections deal with biotechnologically relevant digestive enzymes produced by microbial symbionts in insects.

7.8.1. Cellulases, ligninases, and pectinases

Cellulases (cellulose-hydrolyzing enzymes, including endoglucanases, exoglucanases, and β-glucosidases) are a major group of industrial enzymes with applications in textile processing, paper recycling, and detergent production and in the food industry. Biotechnologically important cellulose-producing fungi and bacteria include the free-living taxa *Trichoderma reesei*, *Humicola insolens, Aspergillus niger, Bacillus subtilis*, and *Clostridium* spp. (Bhat 2000; Phitsuwan *et al.* 2013). However, there are numerous reports on insects hosting symbiotic microorganisms to digest plant fibers, which may yield novel cellulases as well as other biotechnologically important proteins such as carbohydrate binding modules (CBMs) and enzymes of the family AA9 (formerly GH61) that synergistically enhance the efficacy of existing cellulolytic enzymes and have recently been discovered from insect symbionts (Takasuka *et al.* 2013; Poulsen *et al.* 2014). Particularly, termites host an extensively studied and biotechnologically promising gut community of cellulase producers, which can digest up to 74–99 % of the ingested cellulose and about 65– 87 % of the hemicellulose (Breznak and Brune 1994). In addition to the termites' endogenous cellulases (Watanabe and Tokuda 2010), lower termites host cellulolytic protists, while higher termites harbor hindgut bacteria (Spirochetes, Bacteroidetes, Firmicutes, and/or Fibrobacteres) to degrade lignocellulose, with the host enzymes acting on the amorphous regions of cellulose and the symbiotic enzymes targeting the crystalline regions (Brune 2014).

Lignocellulose digestion requires diverse glycoside hydro-lases (GH) to break down the different components of plant cell wall before cellulases can come into play. Current industrial processes use crude extracts of cellulolytic fungi (such as *T. reesei*) not only for economic reasons, but also due to the synergism of the multiple enzymes they contain (Fischer *et al.* 2013). Importantly, termite guts also contain a large diversity of GH enzymes (Poulsen *et al.* 2014); *Nasutitermes* sp. gut symbionts contribute 125 GH5 cellulases, 101 GH10 xylanases, and several GH8, GH9, and GH45 endoglucanases (Shi *et al.* 2013). Symbionts in *Nasutitermes takasagoensis* use the cellulosome (an extracellular multiprotein complex with a single cellulose binding domain) to digest cellulose (Tokuda *et al.* 2005), similar to the biotechnologically important *Clostridium thermocellum*, one of the most efficient cellulolytic bacteria. Similarly, a *Streptomyces* sp. associated with wood wasps secretes multiple enzymes including endo- and exoglucanases, with a high biomass-degrading activity comparable to that of *T. reesei* (Takasuka *et al.*

2013). Recently, cellulases have also been discovered from symbionts associated with pine beetles (Book *et al.* 2014), sugarcane weevils (Rinke *et al.* 2011), and a range of other insects, including Orthoptera, Blattaria, Hemiptera, Coleoptera, Hymenoptera, Lepidoptera, and Diptera (reviewed in Calderón-Cortés *et al.* 2012).

Ligninases are also important enzymes in processing lignocellulose. While animals generally lack these enzymes, several fungi and bacteria are able to degrade lignin, producing veratraldehyde in this process, a flavorant and odorant with a pleasant woody fragrance. Several wood-feeding insects (Asian long-horned beetles, Pacific dampwood termite, fungus-growing termites) host symbiotic soft-rot fungi in their guts (Geib *et al.* 2008), either to enable efficient utilization of cellulose or to degrade lignin itself as a source of nutrition (Hyodo *et al.* 2003), both important biotechnological applications.

Similarly, pectinase, an enzyme that breaks down pectin (a heteropolysaccharide found in plant cell walls), has biotechnological potential in extracting fruit juice (e.g., apple juice) and in wine production. Honey bees host symbiotic Gamma-Proteobacteria that possess the genetic potential for pectin degradation and show in vitro production of pectinase to break down pollen cell walls (Engel and Moran 2012). In leaf-cutting ants, pectinolytic enzymes that are ingested from the fungal cultivar pass unaffected through the ant gut and are finally applied to the plant substrates used for fungal cultivation (Schiøtt *et al.* 2010).

7.8.2. Other digestive and nutritionally important enzymes

Lipases and proteases have important applications in the production of biodiesel (conversion of vegetable oil to alcohol esters), synthetic polymers, pharmaceuticals, agrochemicals, cosmetics, flavoring compounds, as well as in bioremediation such as decontamination of wastewater and oil polluted soils. Currently, *Candida* spp., *Pseudomonas* spp., and *Rhizopus* spp. are important sources of lipase production, but several other microbes are used as well (Pandey *et al.* 1999). Symbiotic microorganisms with potential lipase and/or protease activity have been isolated from insects, including *Bacillus*, *Staphylococcus*, *Pseudomonas*, *Fusarium solani, Candida fermentati, Yarrowia lipolytica*, and yeast-like symbionts from silkworm, long-horned beetles, cigarette beetles, cottony maple scale insects, and burying beetles, respectively (Brues and Glaser 1921; Vega and Dowd 2005; Park *et al.* 2007; Feng *et al.* 2011; Scully *et al.* 2012; Kaltenpoth and Steiger 2014). Unfortunately, however, information on their relevance for host fitness or their potential for biotechnological application remains limited. Extracellular lipase-producing symbionts are especially interesting from a biotechnological perspective, such as lipases secreted by yeast-like symbionts associated with deathwatch beetles, wood-boring beetles, and fruit flies, which play a role in the hosts' nutrition (Gonzalez 2014). Gut bacteria in the soybean-feeding velvet bean caterpillar *Anticarsia gemmatalis* produce proteases ("general" and serine proteases) that could block protease inhibitors present in soybean leaves (Visôtto *et al.* 2009). Basidiomycete fungi associated with ants also secrete proteases that help detoxification and nutrient assimilation (Gonzalez 2014).

In addition to the production of biotechnologically interesting digestive enzymes, insect symbionts have been implicated in the biosynthesis of nutritional compounds of applied value, particularly B vitamins, e.g., in lice, tsetse flies, sharpshooters, and aphids (reviewed in Douglas 2009). However, the inability to isolate and culture these symbionts on artificial media severely limits their biotechnological potential. By contrast, cultivable (and therefore biotechnologically more interesting) B vitamin-producing actinobacterial symbionts are known to occur in firebugs and African cotton stainers (Salem *et al.* 2014),

and culturable yeast-like symbionts provide anobiid beetles and wood wasps with sterols, vitamins, and/or essential amino acids (Pant and Fraenkel 1954; Bismanis 1976).

7.9. POTENTIAL ROLE OF INSECT SYMBIONTS IN BIOREMEDIATION AND DETOXIFICATION

Bioremediation is the elimination, attenuation, or transformation of polluting or contaminating substances by the use of biological processes. In addition to the applied potential, understanding how organisms metabolize such compounds has important implications for understanding ecological adaptation and the evolution of resistance against pesticides.

Insects are exposed to toxic natural products (especially plant secondary metabolites) as well as noxious compounds from human activities (pollutants and insecticides). Detoxifying enzymes providing resistance against both groups of chemicals can be produced by the hosts themselves or by microbial symbionts (Douglas 2013). Evidence for fungal symbionts involved in detoxification across several insect orders was reviewed by Dowd (1992); symbiotic fungi be-longing to the genera *Candida, Aspergillus, Amylostereum*,

Phanerochaete, and *Xylaria* are present in bark beetles, wood wasps, leaf-cutting ants, and various other insects, where they may aid in the degradation of terpenes, tannins, toxic esters, phenolics, chlorinated hydrocarbons, alkaloids, and quinones. For example, mycetomes present between the foregut and midgut of the cigarette beetle *Lasioderma serricorne* contain symbiotic yeasts that produce hydrolytic enzymes acting on toxic phenolic compounds such as tannins, as well as on alkaloid esters (Dowd and Shen 1990). The symbionts are thought to be closely related to plant pathogens that evolved similar mechanisms to overcome plant toxins. Similarly, fenitrothion-degrading bacteria (Burkholderiales) have been isolated from the gut of bean bugs (*Riptortus pedestris*) and stinkbugs (*Leptocorisa chinensis*) and shown to confer insecticide resistance to their hosts (Kikuchi *et al.* 2012). The mountain pine beetle *Dendroctonus ponderosae*, a herbivore of conifers, hosts a bacterial gut community dominated by *Pseudomonas, Rahnella,* and *Burkholderia* that display terpene-degrading genes (Adams *et al.* 2013), which putatively benefit the host in detoxification of conifer defenses. An important bioremediation case study comes from bacterial strains of *Enterobacter* and *Bacillus* that were isolated from the gut of the waxworm *Plodia interpunctella* that naturally feed on beeswax. Within 60 days, these bacteria were capable of degrading 6.1 to 10.7 % of polyethylene—a substrate with long-chain hydrocarbons as in their natural diet—when fed as the sole carbon source to the larvae (Yang *et al.* 2014). The breakdown products were reportedly water soluble, but were not further characterized in the study.

Two detoxifying enzymes with particular application potential in biotechnology are linamarase (β-D-glucosidase) and laccase. Linamarase produced by the bacterial gut community (*Acinetobacter* sp., *Bacillus* sp., *Klebsiella* sp., *Alcaligenes* sp.) of variegated grasshoppers (*Zonocerus variegatus*) acts on the cyanogenic glucosides of their food plants (Idowu *et al.* 2009). This enzyme can be used for the reduction of free cyanide levels in the fermentation of cassava fruits that contain cyanogenic glycosides (Ikediobi and Onyike 1982). Laccases, enzymes that catalyze the oxidation of aromatic compounds (phenols and amino-phenols), are widely used in organic synthesis, bioremediation, textile industry, wine stabilization, and biosensors for immunoassays (Kunamneni *et al.* 2008). In a symbiotic context, these enzymes are produced by fungal symbionts of the leaf-cutting ant *Acromyrmex echinator* (De Fine Licht *et al.* 2013). Interestingly, the symbiont-produced enzyme is consumed by the ants and re-mains active during the passage through the gut, where it is used to detoxify phenolic compounds in the plant material that are

then supplied to the fungus for nutrition (De Fine Licht *et al.* 2013). Actinobacteria isolated from the termite *Amitermes hastatus* gut also showed high laccase activity (Le Roes-Hill *et al.* 2011), highlighting the potential of insect symbionts as producers of biotechnologically relevant detoxifying enzymes.

7.10. Conclusions

Insect symbionts constitute a rich and mostly untapped source of bioactive small molecules as well as digestive enzymes of potential biotechnological value. Even though their exploitation is currently hampered by the unculturability of most symbionts, advances in culturing techniques as well as genomic and genetic tools for the identification and heterologous expression of genes of interest may overcome this hurdle. Furthermore, a large diversity of facultative associates is experimentally and/or genetically tractable and could be of more immediate applied value. In addition to exploiting their metabolic capabilities, insect symbionts can be used to promote insect health as well as to target and control agricultural pest insects and vectors of medically important human diseases in an environmentally friendly way. Overall, increasing research efforts in the areas of insect ecology and symbiosis not only promise to uncover interesting new symbiotic alliances but may also prove valuable in the continued effort to find new sources of biotechnologically important molecules and enzymes. Specifically, targeted searches for compounds with particular applied value (e.g., antibiotics, detoxifying enzymes, cellulases, lipases, etc.) may benefit from being guided by the knowledge on host-symbiont ecology, which has the potential to predict particularly promising systems for exploration.

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7.12. Conflict of interest

The authors declare that they have no competing interests.

7.13. Data accessibility

7.13. REFERENCES

Adams AS, Aylward FO, Adams SM, Erbilgin N, Aukema BH, Currie CR, Suen G, Raffa KF (2013) Mountain pine beetles colonizing historical and naive host trees are associated with a bacterial community highly enriched in genes contributing to terpene metabolism. *Applied and Environmental Microbiology,* 79:3468–3475.

Arakaki N, Noda H, Yamagishi K (2000) Wolbachia-induced parthenogenesis in the egg parasitoid *Telenomus nawai*. *Entomologia Experimentalis et Applicata,* 96:177–184.

Arora AK, Forshaw A, Miller TA, Durvasula R (2015) A delivery system for field application of paratransgenic control. *BMC Biotechnology* ,15: 59.

Atyame CM, Pasteur N, Dumas E, Tortosa P, Tantely ML, Pocquet N, Licciardi S, Bheecarry A, Zumbo B, Weill M, Duron O (2011) Cytoplasmic incompatibility as a means of controlling *Culex pipiens quinquefasciatus* mosquito in the islands of the south-western Indian Ocean. *PLoS Neglected Tropical Diseases,* 5:e1440.

Augustinos AA, Kyritsis GA, Papadopoulos NT, Abd-Alla AMM, Caceres C, Bourtzis K (2015) Exploitation of the medfly gut microbiota for the enhancement of sterile insect technique: use of *Enterobacter* sp. in larval diet-based probiotic applications. *PLoS One,* 10.

Bakri A, Heather N, Hendrichs J, Ferris I (2005) Fifty years of radiation biology in entomology: lessons learned from IDIDAS. *Annal Entomoogicall Society of America,* 98:1–12

Barke J, Seipke RF, Grüschow S, Heavens D, Drou N, Bibb MJ, Goss RJM, Yu DW, Hutchings MI (2010) A mixed community of actinomycetes produce multiple antibiotics for the fungus farming ant *Acromyrmex octospinosus. BMC Biology,* 8:109.

Baumann P (2005) Biology of bacteriocyte-associated endosymbionts of plant sap-sucking insects. *Annual Review in Microbiology,* 59:155–189.

Ben Ami E, Yuval B, Jurkevitch E (2010) Manipulation of the microbiota of mass-reared Mediterranean fruit flies *Ceratitis capitata* (Diptera: Tephritidae) improves sterile male sexual performance. *The ISME J*ournal, 4: 28–37.

Ben Beard C, Cordon-Rosales C, Durvasula RV (2002) Bacterial symbionts of the *Triatominae* and their potential use in control of Chagas disease transmission. *Annual Review in Entomology,* 47:123–141

Bhat M (2000) Cellulases and related enzymes in biotechnology. *Biotechnological Advances*, 18:355–383

Bismanis JE (1976) Endosymbionts of *Sitodrepa panicea. Canadian Journal of Microbiology,* 22:1415–1424

Book AJ, Lewin GR, McDonald BR, Takasuka TE, Doering DT, Adams AS, Blodgett JAV, Clardy J, Raffa KF, Fox BG, Currie CR (2014) Cellulolytic *Streptomyces* strains associated with herbivorous insects share a phylogenetically linked capacity to degrade lignocellulose. *Applied and Environmental Microbiology,* 80:4692–4701.

Bourtzis K (2008) *Wolbachia*-based technologies for insect pest population control. *Advances in Experimental Medicine and Biology,* 627:104–113.

Bourtzis K, Robinson AS (2006) Insect pest control using Wolbachia and/ or radiation. In: Bourtzis K, Miller TA (eds) Insect symbiosis. CRC Press, Boca Raton, FL, pp. 225–246

Bourtzis K, Dobson SL, Xi Z, Rasgon JL, Calvitti M, Moreira LA, Bossin HC, Moretti R, Baton LA, Hughes GL, Mavingui P, Gilles JR (2014) Harnessing mosquito-*Wolbachia* symbiosis for vector and disease control. *Acta Tropica*, 132(Suppl):S150–S163.

Brelsfoard CL, St Clair W, Dobson SL (2009) Integration of irradiation with cytoplasmic incompatibility to facilitate a lymphatic filariasis vector elimination approach. *Parasites and Vectors,* 2:38.

Breznak JA, Brune A (1994) Role of microorganisms in the digestion of lignocellulose by termites. *Annual Review in Entomology,* 39:453–487.

Brown AMV, Huynh LY, Bolender CM, Nelson KG, McCutcheon JP (2014) Population genomics of a symbiont in the early stages of a pest invasion. *Molecular Ecology,* 23:1516–1530.

Brues CT, Glaser RW (1921) A symbiotic fungus occurring in the fat body of *Pulvinaria innumerabilis* Rath. *Biological Bulletin,* 40:299–324.

Brune A (2014) Symbiotic digestion of lignocellulose in termite guts. *Nature Reviews Microbiolgy* 12:168–180.

Calderón-Cortés N, Quesada M, Watanabe H, Cano-Camacho H, Oyama K (2012) Endogenous plant cell wall digestion: a key mechanism in insect evolution. *Annual Review of Ecology, Evolution and Systematics,* 43:45–71

Carina Audisio M, Torres MJ, Sabaté DC, Ibarguren C, Apella MC (2011) Properties of different lactic acid bacteria isolated from *Apis mellifera* L. bee-gut. *Microbiological Research,* 166:1–13.

Carr G, Derbyshire ER, Caldera EJ, Currie CR, Clardy J (2012a) Antibiotic and antimalarial quinones from fungus-growing ant-associated *Pseudonocardia* sp. *Journal of Natural Prodducts,* doi:10.1021/np300380t

Carr G, Poulsen M, Klassen JL, Hou YP, Wyche TP, Bugni TS, Currie CR, Clardy J (2012b) Microtermolides A and B from termite-associated *Streptomyces* sp and structural revision of vinylamycin. *Organic Lett* 14:2822–2825.

Chaves S, Neto M, Tenreiro R (2009) Insect-symbiont systems: from complex relationships to biotechnological applications. *Biotechnology Journal,* 4:1753–1765.

Condon KC, Condon GC, Dafa'alla TH, Fu GL, Phillips CE, Jin L, Gong P, Alphey L (2007) Genetic sexing through the use of Y-linked transgenes. *Insect Biochemistry and Molecular Biology,* 37:1168–1176.

Cornman RS, Tarpy DR, Chen Y, Jeffreys L, Lopez D, Pettis JS, vanEngelsdorp D, Evans D (2012) Pathogen webs in collapsing honey bee colonies. *PLoS One*, 7:e43562.

Crotti E, Balloi A, Hamdi C, Sansonno L, Marzorati M, Gonella E, Favia G, Cherif A, Bandi C, Alma A, Daffonchio D (2012) Microbial symbionts: a resource for the management of insect-related problems. *Microbial Biotechnology,* 5:307–317.

De Fine Licht HH, Schiøtt M, Rogowska-Wrzesinska A, Nygaard S, Roepstorff P, Boomsma JJ (2013) Laccase detoxification mediates the nutritional alliance between leaf-cutting ants and fungus-garden symbionts. *Proceedings of the National Academy of Sciences of the United States of America,* 110:583–587.

De Vooght L, Caljon G, De Ridder K, Van Den Abbeele J (2014) Delivery of a functional anti-trypanosome nanobody in different tsetse fly tissues via a bacterial symbiont, *Sodalis glossinidius*. *Microbial Cell Factories* 13:156.

Dettner K (2011) Potential pharmaceuticals from insects and their co-occurring microorganisms. In: Vilcinskas A (ed) Insect Biotechnology, pp 95–119

Douglas AE (2007) Symbiotic microorganisms: untapped resources for insect pest control. *Trends in Biotechnology,* 25:338–342

Douglas AE (2009) The microbial dimension in insect nutritional ecology. *Functional Ecology,* 23:38–47.

Douglas AE (2013) Microbial brokers of insect-plant interactions revisited. *J Chem Ecol* 39:952–961.

Dowd PF (1992) Insect fungal symbionts: a promising source of detoxifying enzymes. *Journal of Industrial Microbiology and Biotechnology,* 9:149–161

Dowd PF, Shen SK (1990) The contribution of symbiotic yeast to toxin resistance of the cigarette beetle (*Lasioderma serricorne*). *Entomolgia Experimentalis et Applicata,* 56:241–248

Durvasula RV, Gumbs A, Panackal A, Kruglov O, Aksoy S, Merrifield RB, Richards FF, Beard CB (1997) Prevention of insect-borne disease: an approach using transgenic symbiotic bacteria. *Proceedings of the National Academy of Sciences of the United States of America,* 94:3274–3278.

Durvasula RV, Kroger A, Goodwin M, Panackal A, Kruglov O, Taneja J, Gumbs A, Richards FF, Beard CB, Cordon-Rosales C (1999) Strategy for introduction of foreign genes into field populations of Chagas disease vectors. *Annals of the Entomological Society of America,* 92:937–943

Engel P, Moran NA (2012) Functional and evolutionary insights into the simple yet specific gut microbiota of the honey bee from metagenomic analysis. *Gut Microbes* 4:60–65.

Evans JD, Lopez DL (2004) Bacterial probiotics induce an immune response in the honey bee (Hymenoptera: Apidae). *Journal of Economic Entomology,* 97:752–756

Feng W, Wang X-Q, Zhou W, Liu G-Y, Wan Y-J (2011) Isolation and characterization of lipase-producing bacteria in the intestine of the silkworm, *Bombyx mori*, reared on different forage. *Journal of Insect Science* 11.

Fischer R, Ostafe R, Twyman R (2013) Cellulases from insects. In: Vilcinskas A (ed) Yellow Biotechnology II. Springer, Berlin Heidelberg, pp. 51–64

Flórez LV, Biedermann PHW, Engl T, Kaltenpoth M (2015) Defensive symbioses of animals with prokaryotic and eukaryotic microorganisms. *Natural Product Reports,* 32:904–936.

Forsgren E, Olofsson TC, Vasquez A, Fries I (2010) Novel lactic acid bacteria inhibiting *Paenibacillus larvae* in honeybee larvae. *Apidologie* 41:99–108.

Francois IEJA, De Bolle MFC, Dwyer G, Goderis IJWM, Woutors PFJ, Verhaert PD, Proost P, Schaaper WMM, Cammue BPA, Broekaert WF (2002) Transgenic expression in *Arabidopsis* of a polyprotein construct leading to production of two different antimicrobial proteins. *Plant Physiology,* 128:1346–1358.

Fredenhagen A, Tamura SY, Kenny PTM, Komura H, Naya Y, Nakanishi K, Nishiyama K, Sugiura M, Kita H (1987) Andrimid, a new peptide antibiotic produced by an intracellular bacterial symbiont isolated from a brown planthopper. *Journal of the American Chemical Society,* 109:4409–4411

Geib SM, Filley TR, Hatcher PG, Hoover K, Carlson JE, Jimenez-Gasco MM, Nakagawa-Izumi A, Sleighter RL, Tien M (2008) Lignin degradation in wood-feeding insects. *Proceedings of the National Academy of Sciences of the United States of America,* 105: 12932–12937.

Gonzalez F (2014) Symbiosis between yeasts and insects. Introductory Pap Fac Landsc Archit, Hortic Crop Prod Sci 3:1–52

Haeder S, Wirth R, Herz H, Spiteller D (2009) Candicidin-producing *Streptomyces* support leaf-cutting ants to protect their fungus garden against the pathogenic fungus *Escovopsis*. *Proceedings of the National Academy of Sciences of the United States of America,* 106:4742–4746

Himler AG, Adachi-Hagimori T, Bergen JE, Kozuch A, Kelly SE, Tabashnik BE, Chiel E, Duckworth VE, Dennehy TJ, Zchori-Fein E, Hunter MS (2011) Rapid spread of a bacterial symbiont in an invasive whitefly is driven by fitness benefits and female bias. *Science* 332:254–256.

Hoffmann AA, Montgomery BL, Popovici J, Iturbe-Ormaetxe I, Johnson PH, Muzzi F, Greenfield M, Durkan M, Leong YS, Dong Y, Cook H, Axford J, Callahan AG, Kenny N, Omodei C, McGraw EA, Ryan PA, Ritchie SA, Turelli M, O'Neill SL (2011) Successful establishment of *Wolbachia* in *Aedes* populations to suppress dengue trans-mission. *Nature* 476:454–457.

Hosokawa T, Kikuchi Y, Shimada M, Fukatsu T (2007) Obligate symbiont involved in pest status of host insect. *Proceedings of the Royal Society of London B Biological Sciences,* 274: 1979–1984.

Hyodo F, Tayasu I, Inoue T, Azuma JI, Kudo T, Abe T (2003) Differential role of symbiotic fungi in lignin degradation and food provision for fungus-growing termites (Macrotermitinae: Isoptera). *Functional Ecology* 17:186–193. ´

Idowu AB, Edema MO, Oyedepo MT (2009) Extracellular enzyme production by microflora from the gut region of the variegated grass-hopper *Zonocerus variegatus* (Orthoptera: Pyrgomorphidae). *International Journal of Tropical Insect Science,* 29:229–235.

Ikediobi CO, Onyike E (1982) Linamarase activity and detoxification of cassava (*Manihot esculenta*) during fermentation for gari production. *Agricultural and Biological Chemistry,* 46:1667–1669

Juan-Blasco M, San Andrés V, Martinez-Utrillas MA, Argilés R, Pla I, Urbaneja A, Sabater-Muñoz B (2013) Alternatives to gingerroot oil aromatherapy for improved mating performance of sterile *Ceratitis capitata* (Diptera: Tephritidae) males. *Journal of Applied Entomology,* 137:244–251

Jurkevitch E (2011) Riding the Trojan horse: combating pest insects with their own symbionts. *Microbial Biotechnology,* 4:620–627.

Kaltenpoth M (2009) Actinobacteria as mutualists: general healthcare for insects? *Trends in Microbiology* 17:529–535

Kaltenpoth M, Steiger S (2014) Unearthing carrion beetles' microbiome: characterization of bacterial and fungal hindgut communities across the *Silphidae*. *Molecular Ecology,* 23:1251–1267

Kaltenpoth M, Gottler W, Herzner G, Strohm E (2005) Symbiotic bacteria protect wasp larvae from fungal infestation. *Current Biology,* 15:475– 479.

Kaltenpoth M, Roeser-Mueller K, Koehler S, Peterson A, Nechitaylo T, Stubblefield JW, Herzner G, Seger J, Strohm E (2014) Partner choice and fidelity stabilize co-evolution in a Cretaceous-age defensive

symbiosis. *Proceedings of the National Academy of Sciences of the United States of America,* 111:6359–6364.

Kambris Z, Cook PE, Phuc HK, Sinkins SP (2009) Immune activation by life-shortening *Wolbachia* and reduced filarial competence in mosquitoes. *Science* 326:134–136.

Kellner RLL (2001) Suppression of pederin biosynthesis through antibiotic elimination of endosymbionts in *Paederus sabaeus*. *Journal of Insect Physiology,* 47:475–483

Kellner RLL (2002) Molecular identification of an endosymbiotic bacterium associated with pederin biosynthesis in *Paederus sabaeus* (Coleoptera: Staphylinidae). *Insect Biochemistry and Molecular Biology* 32:389–395

Kikuchi Y, Hayatsu M, Hosokawa T, Nagayama A, Tago K, Fukatsu T (2012) Symbiont-mediated insecticide resistance. *Proceedings of the National Academy of Sciences of the United States of America,* 109:8618–8622.

Knipling EF (1955) Possibilities of insect control or eradication through the use of sexually sterile males. *Journal of Economic Entomology,* 48:459–462

Kroiss J, Kaltenpoth M, Schneider B, Schwinger M-G, Hertweck C, Maddula RK, Strohm E, Svatoš A (2010) Symbiotic streptomycetes provide antibiotic combination prophylaxis for wasp offspring. *Nature Chemical Biology,* 6:261–263

Kunamneni A, Plou FJ, Ballesteros A, Alcalde M (2008) Laccases and their applications: a patent review. *Recent Patents on Biotechnology* 2:10–24

Laven H (1967) Eradication of *Culex pipiens fatigans* through cytoplasmic incompatibility. *Nature* 216:383–384.

Le Roes-Hill M, Rohland J, Burton S (2011) Actinobacteria isolated from termite guts as a source of novel oxidative enzymes. *Anton Leeuwenhoek Internation Journal of General and Molecular Microbiology,* 100:589–605.

McMeniman CJ, Lane RV, Cass BN, Fong AWC, Sidhu M, Wang YF, O'Neill SL (2009) Stable introduction of a life-shortening *Wolbachia* infection into the mosquito *Aedes aegypti*. *Science* 323: 141–144.

Moreira LA, Iturbe-Ormaetxe I, Jeffery JA, Lu G, Pyke AT, Hedges LM, Rocha BC, Hall-Mendelin S, Day A, Riegler M, Hugo LE, Johnson KN, Kay BH, McGraw EA, van den Hurk AF, Ryan PA, O'Neill SL (2009) A *Wolbachia* symbiont in *Aedes aegypti* limits infection with dengue, Chikungunya, and Plasmodium. *Cell* 139:1268–1278.

Nakabachi A, Ueoka R, Oshima K, Teta R, Mangoni A, Gurgui M, Oldham NJ, van Echten-Deckert G, Okamura K, Yamamoto K, Inoue H, Ohkuma M, Hongoh Y, S-y M, Hattori M, Piel J, Fukatsu T (2013) Defensive bacteriome symbiont with a drastically reduced genome. *Current Biology,* 23:1478–1484.

Nogge G (1976) Sterility in tsetse flies (*Glossina morsitans* Westwood) caused by loss of symbionts. *Experientia* 32:995–996.

Oh DC, Poulsen M, Currie CR, Clardy J (2009a) Dentigerumycin: a bacterial mediator of an ant-fungus symbiosis. *Nature Chemical Biology,* 5: 391–393

Oh DC, Scott JJ, Currie CR, Clardy J (2009b) Mycangimycin, a polyene peroxide from a mutualist *Streptomyces* sp. *Organic Letters* 11:633–636

Olofsson TC, Vásquez A (2008) Detection and identification of a novel lactic acid bacterial flora within the honey stomach of the honeybee *Apis mellifera. Current Microbiolo*gy57:356–363

Pandey A, Benjamin S, Soccol CR, Nigam P, Krieger N, Soccol VT (1999) The realm of microbial lipases in biotechnology. *Biotechnology and Applied Biochemistry* 29:119–131.

Pant NC, Fraenkel G (1954) Studies on the symbiotic yeasts of two insect species, *Lasioderma serricorne* F. and *Stegobium paniceum L. The Biological Bulletin,* 107:420–432.

Park D-S, Oh H-W, Bae K-S, Kim H-M, Heo S-Y, Kim N-J, Seol K-Y, Park H-Y (2007) Screening of bacteria producing lipase from insect gut: isolation and characterization of a strain, *Burkholderia* sp. HY-10 producing lipase. *Korean Journal of Applied Entomology* 46:131–139

Phitsuwan P, Laohakunjit N, Kerdchoechuen O, Kyu K, Ratanakhanokchai K (2013) Present and potential applications of cellulases in agriculture, biotechnology, and bioenergy. *Folia Microbiologica,* 58:163–176.

Piel J (2002) A polyketide synthase-peptide synthetase gene cluster from an uncultured bacterial symbiont of *Paederus* beetles. *Proceedings of the National Academy of Sciences of the United States of America,* 99:14002–14007

Poulsen M, Hu H, Li C, Chen Z, Xu L, Otani S, Nygaard S, Nobre T, Klaubauf S, Schindler PM, Hauser F, Pan H, Yang Z, Sonnenberg ASM, de Beer ZW, Zhang Y, Wingfield MJ, Grimmelikhuijzen CJP, de Vries RP, Korb J, Aanen DK, Wang J, Boomsma JJ, Zhang G (2014) Complementary symbiont contributions to plant decomposition in a fungus-farming termite. *Proceedings of the National Academy of Sciences of the United States of America* 111: 14500–14505.

Promnuan Y, Kudo T, Chantawannakul P (2009) Actinomycetes isolated from beehives in Thailand. *World Journal of Microbiology and Biotechnology,* 25:1685– 1689.

Ramadhar TR, Beemelmanns C, Currie CR, Clardy J (2014) Bacterial symbionts in agricultural systems provide a strategic source for antibiotic discovery. *Journal of Antibiotics,* 67:53–58.

Rinke R, Costa A, Fonseca F, Almeida L, Júnior ID, Henrique-Silva F (2011) Microbial diversity in the larval gut of field and laboratory populations of the sugarcane weevil *Sphenophorus levis* (Coleoptera, Curculionidae). *Genetics and Molecular Research,* 10:2679–2691

Salem H, Kreutzer E, Sudakaran S, Kaltenpoth M (2013) Actinobacteria as essential symbionts in firebugs and cotton stainers (Hemiptera, Pyrrhocoridae). *Environmental Microbiology* 15:1956–1968.

Salem H, Bauer E, Strauss AS, Vogel H, Marz M, Kaltenpoth M (2014) Vitamin supplementation by gut symbionts ensures metabolic ho-meostasis in an insect host. *Proceedings of the Royal Society of London B-Biological Sciences*, 281.

Salem H, Florez L, Gerardo N, Kaltenpoth M (2015) An out-of-body experience: the extracellular dimension for the transmission of mutualistic bacteria in insects. *Proceedings of the Royal Society of London B-Biological Sciences*

Schiøtt M, Rogowska-Wrzesinska A, Roepstorff P, Boomsma JJ (2010) Leaf-cutting ant fungi produce cell wall degrading pectinase complexes reminiscent of phytopathogenic fungi. *BMC Biology,* 8:156

Schoenian I, Spiteller M, Ghaste M, Wirth R, Herz H, Spiteller D (2011) Chemical basis of the synergism and antagonism in microbial communities in the nests of leaf-cutting ants. *Proceedings of the National Academy of Sciences of the United States of America,* 108:1955–1960.

Scully ED, Hoover K, Carlson J, Tien M, Geib SM (2012) Proteomic analysis of *Fusarium solani* isolated from the Asian longhorned beetle, *Anoplophora glabripennis*. *PLoS One,* 7:e32990

Shi W, Xie S, Chen X, Sun S, Zhou X, Liu L, Gao P, Kyrpides NC, No E-G, Yuan JS (2013) Comparative genomic analysis of the endosymbionts of herbivorous insects reveals eco-environmental adaptations: biotechnology applications. *PLoS Genetics,* 9:e1003131.

Stouthamer R (1993) The use of sexual versus asexual wasps in biological control. *Entomophaga* 38:3–6.

Sudakaran S, Retz F, Kikuchi Y, Kost C, Kaltenpoth M (2015) Evolutionary transition in symbiotic syndromes enabled diversification of phytophagous insects on an imbalanced diet. *The ISME Journal,* 9: 2587–2604.

Sweeny TL, Barr AR (1978) Sex ratio distortion caused by meiotic drive in a mosquito, *Culex pipiens* L. *Genetics,* 88:427–446

Takasuka TE, Book AJ, Lewin GR, Currie CR, Fox BG (2013) Aerobic deconstruction of cellulosic biomass by an insect-associated *Streptomyces*. *Scientific Repports,* 3:1030.

Taracena ML, Oliveira PL, Almendares O, Umana C, Lowenberger C, Dotson EM, Paiva-Silva GO, Pennington PM (2015) Genetically modifying the insect gut microbiota to control Chagas disease vectors through systemic RNAi. *PLoS Neglected Tropical Diseases* 9:e0003358.

Tokuda G, Lo N, Watanabe H (2005) Marked variations in patterns of cellulase activity against crystalline- vs. carboxymethyl-cellulose in the digestive systems of diverse, wood-feeding termites. *Physiological Entomology* 30:372–380.

Tsuchida T, Koga R, Fukatsu T (2004) Host plant specialization governed by facultative symbiont. *Science* 303:1989.

Vega FE, Dowd PF (2005) The role of yeasts as insect endosymbionts. In: Blackwell M, Vega FE (eds) Insect-fungal assocations: ecology and evolution. Oxford University Press, Oxford, pp. 211–243

Visôtto LE, Oliveira MGA, Guedes RNC, Ribon AOB, Good-God PIV (2009) Contribution of gut bacteria to digestion and development of the velvetbean caterpillar, *Anticarsia gemmatalis*. *Journal of Insect Physiology,* 55:185–191

Walker T, Johnson PH, Moreira LA, Iturbe-Ormaetxe I, Frentiu FD, McMeniman CJ, Leong YS, Dong Y, Axford J, Kriesner P, Lloyd AL, Ritchie SA, O'Neill SL, Hoffmann AA (2011) The wMel *Wolbachia* strain blocks dengue and invades caged *Aedes aegypti* populations. *Nature* 476:450–453.

Wang L, Feng Y, Tian J, Xiang M, Sun J, Ding J, Yin W-B, Stadler M, Che Y, Liu X (2015) Farming of a defensive fungal mutualist by an attelabid weevil. *The ISME Journal,* 9:1793–1801.

Watanabe H, Tokuda G (2010) Cellulolytic systems in insects. *Annual Review in Entomology,* 55:609–632

Werren JH (1997) Biology of *Wolbachia*. *Annual Review in Entomology,* 42:587– 609.

Yang J, Yang Y, Wu W-M, Zhao J, Jiang L (2014) Evidence of polyethylene biodegradation by bacterial strains from the guts of plastic-eating waxworms. *Environmental Science and Technology,* 48:13776–13784.

Zabalou S, Riegler M, Theodorakopoulou M, Stauffer C, Savakis C, Bourtzis K (2004) *Wolbachia*-induced cytoplasmic incompatibility as a means for insect pest population control. *Proceedings of the National Academy of Sciences of the United States of America,* 101:15042–15045.

CHAPTER VII

GENERAL DISCUSSION

7.1. THE COMPLEXITY OF CONIFER DEFENSES

Conifers are large, long-lived and stationary organisms and as such have been susceptible to a wide array of both biotic and abiotic stresses throughout their long evolutionary history (Klepzig *et al.* 1995). Herbivorous insects represent one of the most important threats a conifer must face (Keeling & Bohlmann 2006), and thus, trees have evolved mechanical and chemical defensive mechanisms (Huber & Bohlmann 2006) some of which are constitutively expressed while others can be induced upon attack (Litvak & Monson 1998).

Conifer chemical defenses are mainly composed of a complex mixture of terpenoid and phenolic secondary metabolites called oleoresin (Keeling & Bohlmann 2006). Both the qualitative and quantitative composition of these compounds in the resinous mixture is essential in determining the role they play in plant-insect interactions (Langenheim 2003). For instance, the ratio of different classes of compounds regulates the viscosity of resin (Phillips & Croteau 1999). The volatile fraction makes the mixture more fluid, facilitating the flow towards the site of attack, whereas the non-volatile fraction polymerizes easily (Trapp & Croteau 2001), trapping insects and sealing the wound, protecting it from further attack or pathogen invasion.

Terpene profiles in coniferous trees are highly variable across geographical regions (Manninen *et al.* 2002), species (Fäldt *et al.* 2001), genotypes (Martin *et al.* 2003), within the same tree (Jackson *et al.* 1996) and even within the same organ (Litvak & Monson 1998). Terpenoid profiles often correlate with tree resistance against herbivores and pathogens (Mita *et al.* 2002, Raffa & Berryman 1982). For example, the presence of limonene, among other monoterpenes, is associated with Italian stone pine (*Pinus pinea*) resistance against scale insects (Mita *et al.* 2002). Likewise, tolerance of white spruce to the white pine weevil correlates with plant diterpene content (Tomlin *et al.* 2000).

However, the effect that terpenes have on insects that mediates tree resistance is most often unknown. Some compounds are feeding deterrents (Lindgren *et al.* 1996). Others are attractants and repellents, such as the monoterpene limonene, which inhibits the attraction of some pine weevils such *Hylobius abietis* and *H. pinastris* to alpha-pinene, another monoterpene (Nordlander *et al.* 1990). Additionally, some terpenoids are directly toxic to herbivores (Cook & Hain 1988). Spruce bark beetle (*Dendroctonus rufipennis*) survival is reduced by 59% after exposure to limonene for 24 hours (Werner 1995). Likewise, monoterpene concentrations at constitutive expression levels in *Pinus resinosa* kill *Ips pini* individuals within two days, and the induced monoterpene concentration kills 80% of beetles within one day (Raffa & Smalley 1995).

In order to recognize the function that terpenes play in plant-insect interactions, we must understand how conifers produce such a diverse mixture of defensive compounds. Likewise, it is important to explore how insects are affected by these compounds and how they respond to them. The experimental manipulation of plant defenses through genetic engineering of their metabolic pathways is therefore an important tool in answering these questions. For instance, silencing of the geranyl diphosphate synthase in the angiosperm plant *Nicottiana attenuata* resulted in low amounts of 17-hydroxygeranyllinalool diterpenoid

glycoside (HGL-DTG) (Jassbi *et al.* 2008). This change in terpenoid concentration caused tobacco hornworm larvae feeding on these plants to gain three times more weight than those feeding on control plants with high amounts of HGL-DTG (Jassbi *et al.* 2008). These results suggest that this particular HGL-DTG might play a role in plant defense, possibly as a feeding inhibitor.

Along similar lines, in Chapter 3 (Nagel *et al.* 2014) we overexpressed IDS1 in Norway spruce (*Picea abies*) plants. This enzyme mediates the synthesis of geranyl diphosphate (GDP) and geranylgeranyl diphosphate (GGDP), representing a branching point in terpene biosynthesis (see Figure 1 in section 1.6.1), where the pathways diverge into different metabolic routes thereby producing mono-, sesqui-, and diterpenes. Although we observed an increase in IDS1 transcript levels, enzyme activity and GDP and GGDP concentrations in the needles of transgenic plants, we detected no differences in the amount of mono- or diterpenes produced (Chapter 2, Nagel *et al.* 2014). Likewise, there was no difference in the observed amount of other isoprenoids, such as sterols, and pigments like chlorophyll and carotenoids (Chapter 2, Nagel *et al.* 2014). Instead, we detected an increased amount of geranylgeranyl fatty acid esters (Chapter 2, Nagel *et al.* 2014).

Similar compounds to these fatty esters have previously been described in spruce wood (Ekman 1980), although their role remained unexplored. Our results suggest that they could be involved in plant defense against herbivores given that nun moth larvae (*Lymantria monacha*), feeding on transgenic plants needles experienced reduced growth and survival (Chapter 2, Nagel *et al.* 2014). Although the mechanism by which these compounds exert their toxicity is unknown, it could be derived from their hydrolysis inside the insect. This would cause cleavage of the ester bond and the release of geranylgeraniol, which is known to be toxic against *Staphylococcus aureus* (Funari *et al.* 2005, Inoue *et al.* 2005), ants and termites (Lemaire *et al.* 1990). This hypothesis is supported by the high esterase activity present in the gut of the closely related *Lymantria dispar* (Kapin and Ahmad 1980).

Our results show the complexity of terpenoid synthesis pathways and the intricacy of its regulation. Moreover, they also highlight how little we know about plant defensive compounds, as neglected metabolites may play an important role in plant resistance against herbivory.

However, herbivores do not interact with plants alone; they harbor communities of microbes within their digestive system that can also be affected by ingested toxins (Hammer & Bowers 2015). For example, Kohl and Dearing demonstrated that the gut bacterial community of herbivores can be affected by plant secondary metabolites (Kohl & Dearing 2012). Furthermore, it has been suggested that herbivore microbiomes themselves might in some cases be the target of plant toxins (Hammer & Bowers 2015). Therefore, understanding the factors influencing herbivore microbiomes and their interactions with plant defenses is essential in elucidating the range of strategies that herbivores employ to overcome plant toxins.

7.2. GUT MICROBIOTA COMPOSITION IN INSECTS

Although the gut microbiota of insects can harbor fungi, protists and archaea, its major fraction is normally comprised of bacteria (Engel & Moran 2013). Insect bacterial assemblies are highly variable in size, composition, location within the gut and function (Engel & Moran 2013). For example, the number of bacteria in the honey bee gut is estimated to be 10^9 (Martinson *et al.* 2012), whereas *Drosophila*

melanogaster harbors around 10^5 bacterial cells (Ren *et al.* 2007, Ryu *et al.* 2010). Likewise, bacterial diversity is highly variable, with mammals containing thousands of bacterial species in their guts (Ley *et al.* 2006), and insects harboring just a few tens (Colman *et al.* 2012).

Several factors determine the composition of gut bacterial assemblies in insects (Yun *et al.* 2014), including gut structure, physicochemical conditions, and type of food ingested (Dillon & Dillon 2004). Gut structure, for instance, largely determines the size of bacterial communities, being larger in compartmentalized guts such as that of termites (Dillon & Dillon 2004) than in long and narrow guts like those found in aphids or lepidopterans (Engel & Moran 2013).

Gut physicochemical conditions such as pH and oxygen content highly constrain microbial colonization (Engel & Moran 2013, Dillon & Dillon 2004). Some insects feeding on tannin-containing leaves maintain a gut pH of 8, which presumably enhances digestion of these compounds (Berenbaum 1980, Clark 1999). This high pH is thought to select for a particular assembly of microbes able to survive those extreme conditions (Berenbaum 1980). Conversely, carpenter ants ingest formic acid (pH 3.4) released from a gland in their abdomen to acidify their gut, in order to exclude all bacteria except their symbionts, which can stand these acidic conditions (Yong 2016).

Diet can fundamentally affect gut bacterial composition (Colman *et al.* 2012). There is experimental evidence suggesting that changing an insect´s diet can alter itsd gut bacterial assembly (Broderick *et al.* 2004, Sharon *et al.* 2010). Diet could promote the growth of particular bacteria by providing them with a suitable ecological niche thorough appropriate physical, chemical and nutritional conditions (Pernice *et al.* 2014). Additionally, diet could function as a vector for commensal transient bacteria (Pernice *et al.* 2014). The strong influence of diet is shown by the fact that unrelated organisms feeding on similar diets sometimes have similar gut microbial assemblies (Ley *et al.* 2008, Muegge *et al.* 2011). For instance, fungus growing insects such as ants, termites, bark beetles and ambrosia beetles harbor an overlapping microbial community despite belonging to three different insect orders (Aylward *et al.* 2014).

Host phylogeny has also been regarded as one of the forces shaping gut microbial community structure (Colman *et al.* 2012). For example, the microbiota of *Cephalote* ants (Sanders *et al.* 2014), social bees (Engle & Moran 2013), as well as hominids highly correlate with host phylogeny (Ochman *et al.* 2010). However, disentangling whether diet or phylogeny is the driving factor in gut microbial composition is challenging given the diversity of factors that might be involved (Sanders *et al.* 2014). For example, diet may be associated with host phylogeny due to diet-driven evolution of hosts (Coleman *et al.* 2012), as may be the case in phytophagous insects (Farrell 1998). Thus, the relative influence of diet and taxonomy on host gut communities is yet unclear (Coleman *et al.* 2012).

7.2.1. The gut microbiota of conifer-feeding beetles

Similar to most insects (Colman *et al.* 2012, Yun *et al.* 2014), the gut microbiotas of the pine weevil and the parasitic bark beetles *D. punctatus*, *D. valens* and *D. micans* were dominated by Proteobacteria (Chapter 3 and 4; Berasategui *et al.* 2016, Dohet *et al.* 2016).

The most abundant member of the pine weevil microbiome was *Wolbachia*, whereas this bacterium was completely absent from our surveyed bark beetles (Chapter 3 and 4; Berasategui *et al.* 2016, Dohet *et al.* 2016). *Wolbachia* is extremely prevalent in insect populations, infecting around 75% of all known insect species (Hilgenboecker *et al.* 2008). Lachowska and colleagues (2010) estimated that around 40% of central European weevils are infected with this bacterium. Often *Wolbachia* is restricted to the reproductive tissues and is involved in the reproductive manipulation of its host (Werren *et al.* 2008).

However, the presence of *Wolbachia* in the gut of the pine weevil might point towards a nutritional function, as it has been suggested in *Acromyrmex* leaf-cutting ants (Andersen *et al.* 2012). In any case, it is not an obligate symbiont, as our metagenomic analysis of the gut bacterial community in the pine weevil did not retrieve any sequence that was taxonomically assigned to this bacterium (Chapter 4; Berasategui *et al. submitted*).

Excluding *Wolbachia* sequences, the gut communities of both the pine weevil, and bark beetles were dominated by Enterobacteriales (Chapter 3 and 4; Berasategui *et al.* 2016, Dohet *et al.* 2016), which have been previously described as the most prevalent taxa in other bark beetles such as *D. valens*, *D. ponderosae*, *D. frontalis*, *D. rhizophagous* and *I. pini* (Delalibera *et al.* 2007, Morales-Jimenez *et al.* 2009, 2012, Adams *et al.* 2013, Vasanthakumar *et al.* 2006).

Although gut microbiomes in insects can be very variable in composition (Toju & Fukatsu 2011, Zouache *et al.* 2011), stability suggests a functionally relevant community as shown in termites, firebugs and bees (Hongoh *et al.* 2005, Sudakaran *et al.* 2012, Martinson *et al.* 2011). For example, firebugs possess a geographically stable and resilient microbiota with a core comprising *Coriobacterium glomerans*, *Gordonibacer sp* and another two to four bacterial strains (Sudakaran *et al.* 2012), out of which at least *C. glomerans* and/or *Gordonibacter* are essential, as they supplement the insect diet with B vitamins required for development (Salem *et al.* 2014). In the case of pine weevils, we consistently found several members of the community that are closely related to *Erwinia* sp., *Serratia* sp. and *Rahnella* sp. Not only were they present in weevils of every location sampled (Chapter 3, Berastegui *et al.* 2016), but they are also ubiquitous in all parasitic bark beetle species surveyed (Chapter 4, Dohet *et al.* 2016), and have been reported in many other bark beetle species (Adams *et al.* 2013, Vasanthakumar *et al.* 2006, Morales-Jimenez *et al.* 2012, Delalibera *et al.* 2007).

Despite sharing the same dominant taxa at upper and lower taxonomical levels, we found slight differences in the bacterial composition of the pine weevil among locations, which lead to the separation of microbiomes by geographical region (Chapter 3, Berasategui *et al.* 2016). By contrast, gut bacterial communities from the different parasitic bark beetles showed no differences between lab and field collected individuals, nor between species (Chapter 4, Dohet *et al.* 2016). Phloem-feeding beetles have been found to exhibit highly variable intra- and interspecies gut communities (Schloss *et al.* 2006, Grunwald *et al.* 2010). Thus, the stability reported here is noteworthy and strongly suggests an important function of the microbiota.

Both diet and taxonomy can influence microbial community composition (Colman *et al.* 2012). Our meta-analysis of the gut microbiota of conifer-feeding insects (Chapter 2, Berasategui *et al.* 2016) suggests that conifer-feeding beetles share a core set of microbes, all of which belong to the Enterobacteriaceae (and possibly *Pseudomonas*), that is absent from related beetles feeding on non-coniferous substrates. Within curculionids, this shared microbiota has been reported in 10 different species, spanning three genera: *H. abietis, D. valens, D. micans, D. punctatus, D. ponderosae, D. rhizophagous, D. frontalis, D. armandi, D. rufipennis* and *I. pini* (Adams *et al.* 2013, Vasanthakumar *et al.* 2006, Morales-Jimenez *et al.* 2012, Delalibera *et al.* 2007, Berasategui *et al.* 2016, Dohet *et al.* 2016, Mason *et al.* 2016). Additionally, some members of this gut community have also been observed to occur together in the nematode (*Bursaphelenchus xylophilus*), its cerambycid beetle vector (*Monochamus galloprovincialis*), a wood boring wasp (*Sirex noctioa*) and a sawfly (*Neodiprion abietis*) (Vicente *et al.* 2013, Adams *et al.* 2011, Whittome *et al.* 2007), all of them exploiting conifers as an ecological niche. This suggests that the gut community assembly of conifer-feeding insects is likely driven by the diet, rather than phylogeny. Moreover, the stability of this microbiota across different orders, species, genera and geographical regions suggest that they are of importance to their host and most likely represent an adaptation to exploit conifers as a food source.

7.3. ACQUISITION OF GUT BACTERIAL SYMBIONTS IN INSECTS

Due to the many benefits that symbionts can contribute to their hosts, transmission to the next generation must be insured. Here it is important to distinguish transmission modes (vertical vs. horizontal) from transmission routes (extracellular vs. intracellular) (Salem *et al.* 2015). Often intracellular bacteria, those that spend their lifetime inside their host cells, are vertically transmitted from mother to offspring (Bright & Bulgheresi 2010).

However, extracellular symbionts, those that can spend at least part of their lifetime outside their host or host cells, can be transmitted through a variety of different strategies: coprophagy, environmental determination, social acquisition, smearing of brood cell or egg surface, infection via jelly-like secretions or capsule transmission (extensively reviewed in Salem *et al.* 2015).

7.3.1. The specific case of conifer-feeding beetles

No study to date has focused on the mode and route of transmission of bacterial symbionts in the pine weevil. However, we can speculate based on prior observations. Behavioral studies have shown that female pine weevils lay eggs in carved galleries within conifer bark, deposit feces adjacent to each egg, they push the egg and feces back into the gallery and then block the entrance hole with a mixture of chewed bark and feces (Borg-Karlson *et al.* 2006). This behavior has also been shown in other weevils (Wen *et al.* 2004, Wallace & Sullivan, 1985; Langor & Williams, 1998; Zhang *et al.* 2004, Stansly & Cate, 1984). Borg-Karlson and colleages (2006) demonstrated that this behavior protects the eggs against feeding conspecifics as fungi in the feces release anti-feeding volatiles. This behavior could have been coopted by the insect in order to transmit its gut symbionts, resembling egg smearing or oviposition site inoculation as seen in other insects (Salem *et al* 2015).

Likewise, little is known about bacterial symbiont transmission in bark beetles. Some studies report the presence of bacteria in bark-beetle mycangia that can be transmitted vertically (Hulcr *et al.* 2012). However, the gut bacterial composition of bark beetles and their host trees is very similar (Adams *et al.* 2013), suggesting that although vertical transmission cannot be ruled out, horizontal acquisition of symbionts is very likely. Moreover, Mason and colleagues (2016) found that although the microbiome composition of red pine trees, bark beetles and bark beetle galleries are very similar, bacterial abundances are much lower in the former. The authors propose two non-mutually exclusive hypotheses to explain this pattern. First, they suggest that bark beetles must vector this bacterial community (Mason *et al.* 2016). And, second, they propose that the community may be already present in the bark, albeit in low abundance, but only thrives in a beetle or beetle-engineered environment (Mason *et al.* 2016).

Given the overlapping bacterial assemblies among many bark beetle species and the pine weevil (as well as other conifer-feeding insects), horizontal acquisition of symbionts from the environment and further colonization of the insect gut seems more likely. Also, Xu and colleagues (2015) showed that *Erwinia* sp. increases its abundance in the presence of terpenoids within the gut of a bark beetle. However, *Rahnella* sp, *Serratia* sp. and *Erwinia* sp. are close relatives to plant pathogens and could be using conifer-feeding insects as vectors. For instance, the whitefly *Bemisia tabaci* harbors in its hemolymph the plant pathogen *Rickettsia*, and can vector it from plant to plant (Caspi-Fluger *et al.* 2012).

Although microbes that are acquired from the environment are often considered transient and commensal, they can play a central role in insect ecology. For instance, the bean bug *Riptortus pedestris* acquires its gut symbiont *Burkholderia* horizontally from the environment every generation (Kikuchi *et al.* 2007), and benefits through increased body size and higher survival rates. In fact, environmentally acquired bacteria can provide immediate benefits to their host upon establishment. In fields that have been sprayed with an insecticide, the bean bug can form a symbiotic association with an environmentally acquired insecticide-resistant *Burkholderia*, acquiring rapid resistance itself (Kikuchi *et al.* 2012).

The converged microbiota of conifer-feeding insects suggest it might possess functional traits that are positively selected to inhabit a coniferous environment. Furthermore, the acquisition of this particular bacterial assembly could provide pine weevils and bark beetles with an immediate benefit to exploit conifers.

7.4. ROLES OF GUT SYMBIONTS IN INSECT-MICROBE ASSOCIATIONS

Insect symbionts are known to play a diversity of roles with beneficial functions for their hosts. These range from the supplementation of nutrients and degradation of complex polymers or toxins, to their involvement in developmental processes, immune priming of their host and protection against pathogens or parasites (Engel & Moran 2015).

7.4.1. Roles in development

Although nutritional supplementation (see section 7.3.3) affects insect development and fitness by facilitating the assimilation of essential nutrients, gut symbionts can also affect developmental processes directly (Engel & Moran 2013). For instance, the gut microbiota of *D. melanogaster* influences gut morphology through changes in the epithelial renewal rate, cellular spacing and the composition of different types of cells in the gut epithelium (Broderick *et al.* 2014).

7.4.2. Roles in protection

Symbiotic gut bacteria (either commensal or mutualistic) can be involved in host resistance against parasites (Engel & Moran 2013). Several mechanisms may be involved such as the production of antibacterial compounds (Volaard & Clasener 1994), immune priming (Ivanov *et al.* 2009, Stecher & Hardt 2011), nutrient competition or niche occupation (Engel & Moran 2013).

An *Enterobacter* strain isolated from the gut of wild mosquitos confers its host with resistance against the parasite *Plasmodium falciparium*. It is thought that this bacterium produces reactive oxygen species (ROS) in the gut that directly affects the parasite (Cirimotich *et al.* 2011).

Higher diversity in the composition of gut bacterial communities seem to be related to higher resistance against pathogens (Engel & Moran 2013). Desert locusts (*Schistocerca gregaria)* with highly diverse communities are more resistant to the pathogen *Serratia marcescens*, than those with less diverse communities (Dillon *et al.* 2005).

7.4.3. Roles on nutrition

Nutrient provisioning

Insects exploit a wide variety of ecological niches that are often poor in nutrients (e.g. blood and phloem) and high in complex polymers or chemical defenses (Engel & Moran 2013). Many insects harbor endosymbionts in specialized organs that supplement their diet with essential nutrients. Aphids for instance, harbor the intracellular bacterium *Buchnera* in specialized cells called bacteriocytes. Aphids feed on plant phloem, which is deficient in essential amino acids and some vitamins, and rely on their obligate symbionts for supplementation of their diet (Douglas, 2009).

However, extracellular mutualists, such as gut symbionts can also contribute to diet supplementation in insects. Cotton stainers (*Dysdercus fasciatus*, Hemiptera) feed on the vitamin deficient seeds of Malvales plants (Ahmad and Schaeffer, 1987, Whitsitt, 1933). These insects are capable of exploiting their food source due to the vitamin B supplementing capacities of their gut symbionts (Salem *et al.* 2014). Likewise, the gut symbionts of the blood-feeding insect *Rhodnius prolixus* and plataspid stinkbugs provide their host with B vitamins (Eichler & Schaoub 2002), and essential amino acids (Nikoh *et al.* 2011), respectively.

Symbiont-mediated nutrient provisioning in insects is not restricted to the synthesis of compounds but also involves degradation of recalcitrant polymers. In addition to their own cellulases (Watanabe *et al.* 1998), lower termites are associated with protists that degrade cellulose; whereas higher termites are usually associated with bacteria that perform the same function (Brune & Dietrich 2015), making more carbon available for their host.

Degradation of plant defenses

For many decades, evidence of symbiotic degradation of plant secondary metabolites remained elusive, with the only example of the cigarette beetle *Lasioderma serricorne* and its symbiont *Symbiotaphrina kochi*. This yeast is able to degrade some plant chemicals and xenobiotics in addition to supplementing the host's diet with amino acids, vitamins and sterols (Dowd and Shen 1990, Dowd 1989). Nowadays, there is a growing body of evidence demonstrating that insect symbionts can help their hosts to exploit heavily chemically defended plants through manipulation of plant physiology, inhibition of the synthesis of plant defensive compounds (Chung *et al.* 2013, Barr *et al.* 2010) or facilitating the degradation of defenses once synthesized (Ceja-Navarro *et al.* 2015), as it has been presented in section 1.3.

7.5. INSECT ADAPTATIONS TO EXPLOIT CONIFERS AS A FOOD SOURCE

Conifer-feeding insects have evolved different strategies to cope with the low nutritional value of coniferous tissues. For example, the bark beetle *Ips grandicollis* circumvents low concentrations of nutrients by consuming more phloem than beetles that harbor symbiotic fungi (Ayres *et al.* 2000). Likewise, other insects have evolved diverse strategies to cope with the high concentration of chemical defenses in conifers. For instance, it is well known that several bark beetle species are able to colonize healthy trees by exerting mass attacks, exhausting tree chemical defenses and eventually killing them

(Berryman 1976). Larvae of *D. valens*, *D. micans* and *D. terebrans* feed under the bark of trees in one continuous front, possibly outrunning tree-induced defenses (Gregoire *et al.* 1981, Deneubourg *et al.* 1990). Likewise, tiger moths consume only the upper portions of needles which are lower in monoterpene concentrations than the basal fraction (Litvak & Monson 1998). Also, sawflies feed gregariously to consume conifer needles before plant defenses are induced, or cut the resin ducts of needles before feeding in order to partially release plant toxins (McCullough & Wagner 1993). Additionally, these insects can also sequester conifer resins and employ them against predators (Codella & Raffa 1995).

7.5.1. The role of gut symbionts in conifer-feeding weevils

Whereas many insects rely on their intrinsic capabilities to deal with the low nutritional value and toxic nature of their host plants, others establish symbiotic interactions with microbes that aid in the supplementation of limiting nutrients or in the catabolism of plant defenses.

Nutritional supplementation

Often, bark beetle species associate with fungi that provide the insect with assimilated nitrogen (Six & Paine 1998, Ayres *et al.* 2000, Bleiker & Six 2007) through the translocation of this nutrient from the sapwood to the bark and phloem, thereby increasing its availability by 40% (Bleiker & Six 2007). Others, such as *D. ponderosae* obtains sterols they need for reproduction from ophiostomatoid fungi (Six & Paine 1998, Bentz & Six 2006).

In addition to fungi, bacteria could also be involved in the supplementation of nutrients to conifer-feeding insects. Species of *Pantoea*, *Rahnella* and *Serratia*, which were frequently associated with the pine weevil and with many different bark beetle species, are known to fix nitrogen in plant-microbe interactions (Vasanthakumar *et al.* 2006, Morales-Jimenez *et al.* 2009, Behar *et al.* 2005) and could potentially play the same role for conifer-feeding insects. *Rahnella* sp. have also been reported as uricolytic and could be involved in nitrogen recycling (Morales Jimenez *et al.* 2013). Other members of the microbial community are able to break down cellulose (Morales Jiménez *et al.* 2012).

Degradation of plant defenses

Symbiotic fungi are thought to be involved in insect resistance to terpenoids, although the role they may play in helping the insect exploit their ecological niches has recently been questioned (Six & Wingfield 2011). *Grossmania clavigera*, a bark beetle symbiont, can excrete terpenoids outside its cells via ABC transporters (Wang *et al.* 2013) and can exploit monoterpenes as a carbon source (DiGuistini *et al.* 2011). This symbiont also shows an upregulation of P450 and O-methyltransferases genes upon treatment with terpenoids (DiGuistini *et al.* 2011). Both groups of enzymes are known to detoxify several plant secondary metabolites (Wöll *et al.* 2013, Feltrer *et al.* 2010). However, excreted terpenoids would still remain in the environment and be susceptible of harming bark beetles. Thus how the fungal resistance mechanisms affect the insect host remains unknown.

Even less is known about the contributions of bacterial symbionts of conifer-feeding insects towards terpenoid degradation. Although bacteria from the gut of *D. valens* are able to degrade mono and diterpenes *in vitro* (Boone *et al.* 2013), little is known about the contributions of bacterial symbionts of conifer-feeding insects towards terpenoid degradation and host fitness. Given that the presence of

conserved microbial assemblages often suggests an important functional role for their host (Hongoh *et al.* 2005, Sudakaran *et al.* 2012, Martinson *et al.* 2011), the convergent microbiotas found in conifer-feeding beetles (Chapter 3, Berasategui *et al.* 2016; Chapter 4, Dohet *et al.* 2016), represent likely candidates to mediate host resistance against terpenoids.

Our inference of the metabolic capabilities of the pine weevil´s microbial assembly using PICRUSt revealed a high number of putative genes involved in xenobiotic degradation (Chapter 3, Berasategui *et al.* 2016). Our analysis of the bacterial metagenome further corroborated these data, showing the presence of an almost complete gene cluster (*dit* gene cluster) involved in diterpene degradation (Chapter 5, Berasategui *et al. in preparation*). This gene cluster was already described in free-living *Pseudomonas abietaniphila BKM-9* (Martin & Mohn 2000) and *Burkholderia xenovorans* LB4000 (Smith *et al.* 2007). Interestingly, it has also been found in the bacterial metagenome of the bark beetle *Dendroctonus ponderosae* (Adams *et al.* 2013) as well as in the resin of *Pinus sylvestris* and the galls formed by the moth *Retinia resinella* in the same host plant (Vilanova *et al.* 2014).

Despite our failure to detect all genes in the *dit* cluster in the present analyses, catabolism of terpenes is still possible given that studies demonstrate that not all genes are necessary for terpene degradation (Martin & Mohn 2000, Smith *et al.* 2004, 2007). For example, knocking out ditR did not affect growth of *P. abietaniphila* BKME-9 on diterpene-containing media (Martin & Mohn 2000). Knocking out ditQ, does impair growth of *P. abietaniphila* on dehydroabietic acid, but not on abietic acid (Smith *et al.* 2004). Interestingly, ditI, ditH and ditF, essential genes for diterpene degradation, are all present in our metagenomics analysis of the pine weevil´s gut microbiota (Chapter 5, Berasategui *et al. in preparation*). Taxonomical binning of the *dit* gene cluster sequences revealed that most of them were assigned to members of the Enterobacteriaceae family (Chapter 5, Berasategui *et al. in preparation*). Upon antibiotic treatment, the number of *dit* genes present in in the pine weevil bacterial metagenome dramatically decreased, with only one gene remaining (Chapter 5 Berastegui *et al. in preparation*). Likewise, the Enterobacteriaceae family was the most affected by the antibiotic treatment, with its abundance in the insect gut being remarkably reduced. Taken together, these results suggest that members of the Enterobacteriaceae have the potential to degrade diterpenes. Adams and colleagues (2013) also found some of these genes to be assigned to members of the Enterobacteriaceae in the metagenome of the mountain pine beetle, especially to *Yersinia*, *Rahnella* and *Erwinia*, members of the core microbiota in the pine weevil that are repeatedly found in bark beetles (Chapter 3 and 4, Berasategui *et al.* 2016, Dohet *et al.* 2016).

In vitro assays demonstrate that the microbial community of the pine weevil is indeed able to degrade diterpenes in liquid media, reducing, for instance, DHAA concentration by 20% within 5 days (Chapter 5, Berasategui *et al. in preparation*). This is concordant with studies by Boone and colleagues (2013), where they showed that members of the gut microbial assembly of *D. valens* were also able to degrade both mono- and diterpenes *in vitro*. Furthermore, our bioassays, show that the catabolism of terpenes also occurs in the pine weevil *in vivo.* After passage through the gut, the concentration of total diterpenes in feces was reduced by 80% relative to ingested pine tissue in just 16 hours (Chapter 5, Berasategui *et al. in preparation*). Weevil treatment with antibiotics reduced the catabolic ability of the microbial assembly, which led to an increase in the concentration of both mono- and diterpenes in the weevil´s feces (Chapter 5, Berasategui *et al. in preparation*). Upon reinfection of the weevil with their native microbiota, terpene degradation was rescued and the amount detected in the feces was greatly reduced (Chapter 5, Berasategui *et al. in preparation*). These results suggest that the degradation of terpenes observed in the pine weevil is mediated, at least in part, by its gut micriobiota.

7.5.2. Effect of symbiont-mediated terpene degradation on host fitness

In bark-beetles it is unknown how symbiont-mediated degradation of terpenes affects insect fitness. However, the pine weevil seems to benefit from this process. Our bioassays revealed that pine weevils feeding on terpene-containing diet had their fitness significantly enhanced through higher fecundity and hatching rates when they harbored their native gut microbiota (Chapter 5, Berasategui *et al. in preparation*). We did not observe this fitness benefit when the weevils were not feeding on terpenes and their microbiota was not present.

The mechanism by which symbionts exert this benefit on weevils remains unclear. It has been suggested that the low nutritional value and high amounts of defensive compounds of conifers could affect the pine weevil´s fecundity due to differences in the resources it can allocate to egg production (Wainhouse *et al.* 2001). However, nutritional supplementation of essential nutrients by the symbionts does not seem a likely explanation for the observed fitness gain, since our experiments were performed on a rich artificial diet that would have likely obscured the nutritional role of symbionts.

If pine weevils select their microbial partners based on their ability to degrade terpenoids, two scenarios are possible. First, the breakdown products could be used as nutrients, but the same logic as above applies. Second, the microbial assembly could degrade terpenoids to help weevils overcome their toxicity. This possibility may seem unlikely given that weevils devoid of symbionts do survive, lay eggs, and the eggs hatch, which points towards the insect having an intrinsic mechanism to overcome terpenes. However, often insect-driven counter adaptations against plant secondary metabolites are not completely effective (Parr and Thurston 1972; Agrawal *et al.* 2012; Richards *et al.* 2012), and insects must combine more than one strategy to overcome plant defenses (Despres *et al.* 2007). For example, monarch butterfly caterpillars (*Danaus plexippus*) employ four different strategies to cope with milkweed chemical defenses: vein cutting (Helmus *et al.* 2005), sequestration (Nishida 2002), metabolism of cardenolides through aldehyde reductase (Marty *et al.* 1984) and target site mutation (Holzinger *et al.* 1996).

Therefore, it is highly likely that symbiont-mediated degradation of terpenoids benefits the pine weevil by enhancing the intrinsic resistance mechanism of the pine weevil, as has been previously suggested for bark beetles (Raffa 2013). Given the ease of symbiont acquisition from the environment due to their ubiquity (Mason *et al.* 2016) and the different abilities they have in degrading particular compounds (Boone *et al.* 2013), they represent an interesting and accessible pool of terpene-degrading activity, especially considering the variability of terpenoid profiles present in trees (Manninen *et al.* 2002, Fäldt *et al.* 2010, Martin *et al.* 2003, Jackson *et al.* 1996, Litvak & Monson 1998), Symbionts could provide the insect host with the plasticity to overcome plant defenses.

7.6. EXPLOITING SYMBIOSES FOR BIOTECHNOLOGICAL APPLICATION

Our knowledge of microbial ecology and metabolism can help us to predict the presence of particular enzymes or molecules in specific systems and thus allow targeted studies to search for compounds of applied value.

For instance, *Taq* polymerase, the enzyme that is commonly used to amplify DNA in laboratories around the world was isolated from the free living, geyser-inhabiting and thermophilic bacteria *Thermus aquaticus* (Chien *et al.* 1976). At the time, rudimentary DNA amplification required the addition of fresh enzyme after every amplification cycle since the high temperatures needed to separate DNA strands, also

denaturalized polymerases (Kleppe *et al.* 1971). Given that *T. aquaticus* lives at extreme temperatures its polymerase was more stable at high temperatures (80°C), which allowed performing several cycles of DNA amplification with just one addition of polymerase, giving birth to what we know as PCR (polymerase chain reaction) (Saiki *et al.* 1988). Nowadays, *Taq* polymerase has been cloned, modified, and mass-produced to satisfy worldwide demand (Lawyer *et al.* 1993).

In contrast to free-living microbes, symbiont-produced products have not only been optimized by natural selection for their efficiency, but also to be harmless to their host (Flórez *et al.* 2015). Consequently, these compounds are of great interest given that they open an opportunity for use on eukaryotes while avoiding harmful secondary effects (Chapter 6, Berasategui *et al.* 2015).

Given the current pace at which many human pathogens are evolving resistance against all known antibiotics, the discovery of new active molecules targeting bacteria and other pathogens is essential for human health. In the search for such molecules, attention should be drawn to symbiotic systems in which pathogen attack is likely, such as developing larvae or fungal gardens of insects. Wasp larvae from the genus *Philanthus* are protected against pathogens by symbiotic bacteria (Candidatus *Streptomyces philanthi*) woven in their cocoon silk that produce streptochlorin and a mixture of piericidins (Kaltenpoth *et al.* 2005, Kroiss *et al.* 2010, Kaltenpoth *et al.* 2014). Likewise, fungal gardens of fungus growing insects are highly susceptible to pathogen infection, which make these systems very promising sources of bioactive molecules (Ramadhar *et al.* 2014). In fact, a number of fungus-growing ants harbor actinobacterial symbionts that produce a variety of antimicrobial compounds (Oh *et al.* 2009, Barke *et al.* 2010, Haeder *et al.* 2009, Holmes *et al.* 2016).

Symbiotic microbes are also often involved in the degradation of recalcitrant compounds in their host diets (Douglas 2009), and thus their digestive enzymes (e.g. cellulases, ligninases, pectinases) could be of interest for degradation, recycling or bioremediation (Chapter 6, Berasategui *et al.* 2015)

In addition to symbiont-produced products, our knowledge on the ecology of symbiotic interactions can be exploited to control agricultural pests or vector borne diseases (Chapter 7, Berasategui *et al.* 2015). Some symbionts mediate the pest status of their insect hosts (Hosokawa *et al.* 2007). The shieldbug *Megacopta cribaria*, a legume specialist, suffer reduced survival and reproductive output when feeding on soybeans. However, its fitness is rescued when the insect´s symbionts were replaced with symbionts of the soybean specialist *Megacopta punctatissima* (Hosokawa *et al.* 2007). Disruption of such mutualism could lead to the control of this particular pest. Other mechanisms of biological control through the exploitation of symbioses are the use of bacterial strains that induce parthenogenesis in parasitoids (Arakaki *et al.* 2000, Bourtzis 2008), and the putative genetic transformation of plants to produce symbiont-targeted antimicrobial peptides (Francois *et al.* 2002) (Chapter 6, Berasategui *et al.* 2015).

Some insects are vectors to human pathogens such as malaria, dengue and yellow fever. The use of microbial symbionts to reduce the prevalence of these diseases could be achieved by genetically modifying the symbionts to produce antiparasitic compounds in a process that is known as paratransgenesis. For example, *Rhodnius prolixus* is a vector to *Trypanosoma cruzi*, a protozoan that causes Chagas disease. Transmission of *T. cruzi* has been reduced in field trials due to the genetic manipulation of the gut symbiont of *R. prolixus* to produce anti-trypanosomal molecules (Ben Beard *et al.* 2002, Durvasula *et al.* 1997, 1999). Likewise, insects could be infected with microbes such as *Wolbachia* that reduce their lifespan, fertility or susceptibility to the pathogen (Kambris *et al.* 2007, McMeniman *et al* 2009, Chapter 6, Berasategui *et al.* 2015).

Another *Wolbachia*-based strategy to control insect pests and vector-borne diseases is the so called incompatible insect technique (IIT). It relies on *Wolbachia*-induced cytoplasmic incompatibility (CI). In its simplest form, CI results in the death of insect embryos resulting from matings between *Wolbachia*-

infected males and uninfected females (Werren 1997). Thus, the mass release of *Wolbachia*-infected males that are incompatible with wild females results in lower female fertility and ultimately, the suppression of insect populations (Bourtzis *et al.* 2014).

Lastly, gut symbionts could be employed as probiotics for economically important insects such as honey bees (Chapter 6, Berasategui *et al.* 2015). These insects are highly threatened by a combination of pesticide use and infection by natural pathogens (Comman *et al.* 2012). It has been shown that acid lactic bacteria isolated from the gut of honey bees (Oloffson & Vásquez 2008), can enhance honey bee immunity reducing pathogen infection (Fosgren *et al.* 2010). The use of gut symbionts may thus represent an interesting way to improve health and fitness of economically and agriculturally important insects.

The immediate application of symbiosis or symbiont-derived products in biotechnology is often hampered by the difficulty to culture most symbionts. However, recent development of new culturing techniques as well as of genomic and genetic tools for the identification and heterologous expression of genes of interest may circumvent this problem. Likewise, further research in the field of symbiosis, may reveal symbiotic interactions that may be more experimentally and or genetically tractable and could be of more immediate applied value (Chapter 6, Berasategui *et al.* 2015).

7.7. CONCLUSION

The results of this thesis stress the complexity of conifer defenses, their synthesis, regulation and degradation by insect gut symbionts. It was demonstrated that the overexpression of a key enzyme in the biosynthesis of terpenes does not result in the overproduction of these compounds, but rather in an unexpected production of geranyl fatty esters that also function in plant defense. These results highlight the importance of further research in this area, given that overlooked metabolites may play an important role in plant defense.

We also reveal that conifer-feeding insects in general, and the pine weevil and bark beetles in particular, share a conserved gut bacterial assembly composed of members of the Enterobacteriaceae family that are closely related to *Rahnella*, *Serratia*, *Erwinia* and *Yersinia*. The stability and convergence of this microbial composition is highly suggestive of its functional importance.

Our inference of the bacterial metagenome of the pine weevil illustrated an enrichment of genes involved in terpenoid degradation. Our analysis of the metagenome further corroborated this by revealing the existence of an almost complete gene cluster involved in terpenoid catabolism. Our bioassays and chemical analysis highlight the ability of the gut microbiota of the pine weevil to degrade terpenoids both *in vitro* and *in vivo.* Likewise, they demonstrate that the symbiont-mediated degradation of terpenoids benefits insect fitness through higher fecundity and hatching rates, possibly by contributing to the intrinsic ability of the weevil to resist low concentration of terpenes. However, the exact mechanism remains unclear.

As a result of the diversity of metabolic capabilities of insect symbionts and their role in insect ecology, microbial mutualists represent a mostly unexplored source of molecules and digestive enzymes with potential value for biotechnology. Additionally, symbionts could be used to enhance insect health as well as to control vector-borne diseases and agricultural pests. Broadening our knowledge on symbiotic interactions will not only help us predict new systems for exploration, but will also prove to be essential in our quest to find new sources of molecules with biotechnological value.

Terpenes mediate a major fraction of the interactions between coniferous trees and herbivorous insects. In this thesis we expanded current knowledge on how plant defenses affect insect ecology and the range of counter adaptations that insects have evolved against their toxins. Our results further support the "gut microbial facilitation hypothesis" (Hammer and Bowers 2015) which proposes that variation in the symbiotic gut communities of insects leads to variation in the ability of different herbivores to exploit chemically defended plants.

7.8. REFERENCES

Adams SA, Jordan MS, Adams SM, Suen G, Goodwin LY, Davenport KW, Currie CR, Raffa K (2011) Cellulose-degrading bacteria associated with the woodwasp *Sirex noctilio. The ISME Journal* 5, 1323-1331.

Adams AS, Aylward FO, Adams SM et al (2013) Mountain pine beetles colonizing historical and naive host trees are associated with a bacterial community highly enriched in genes contributing to terpene metabolism. *Applied and Environmental Microbiology,* 79:3468–3475.

Agrawal AA, Petschenka G, Bingham RA, Weber MG, Rasmann S (2012). Toxic cardenolides: chemical ecology and coevolution of specialized plant-herbivore interactions. *New Phytologist*, 194: 28-45.

Ahmad, I. y C. W. Schaefer. (1987). Food plants and feeding biology of the Pyrrhocoroidea (Hemiptera). *Phytophaga,* 1:75-92.

Andersen SB, Boye M, Nash DR, Boomsma JJ (2012) Dynamic *Wolbachia* prevalence in *Acromyrmex* leaf-cutting ants: potential for a nutritional symbiosis. *Journal of Evolutionary Biology*, 25, 1340–1350.

Anderson RS (1995) The natural classification of the families of Coleoptera (Nathan Lloyd, London).

Arakaki N, Noda H, Yamagishi K (2000) *Wolbachia*-induced parthenogenesis in the egg parasitoid *Telenomus nawai. Entomol Exp Appl* 96:177–184.

Aylward FO, Suen G, Biedermann PHW *et al.* (2014) Convergent bacterial microbiotas in the fungal agricultural systems of insects. *mBio*, 5 e02077-14.

Ayres MP, Wilkens RT, Ruel JJ, Lombardero MJ, Vallery E (2000) Nitrogen budgets of phloem-feeding bark beetles with and without symbiotic fungi. *Ecology*, 81, 2198–2210.

Barke J, Seipke RF, Grüschow S, Heavens D, Drou N, Bibb MJ, Goss RJM, Yu DW, Hutchings MI (2010) A mixed community of actinomycetes produce multiple antibiotics for the fungus farming ant *Acromyrmex* octospinosus. *BMC Biology,* 8:109.

Barr KL, Hearne LB, Briesacher S, Clark TL, Davis GE (2010) Microbial symbionts in insects influence down-regulation of defense genes in maize. PLoS ONE, 5(6): e11339.

Behar A, Yuval B, Jurkevitch E (2005) Enterobacteria-mediated nitrogen fixation in natural populations of the fruit fly *Ceratitis capitata*. *Molecular Ecology*, 14, 2637–2643.

Ben Beard C, Cordon-Rosales C, Durvasula RV (2002) Bacterial symbionts of the Triatominae and their potential use in control of Chagas disease transmission. *Annual Review in Entomology,* 47:123–141.

Bentz BJ, Six DL (2006) Ergosterol content of fungi associated with *Dendroctonus ponderosae* and *Dendroctonus rufipennis* (Coleoptera: Curculionidae, Scolytinae). Annals of the *Entomological Society of America*, 99, 189–194.

Berasategui A, Shukla S, Salem H, Kaltenpoth M (2015) Potential applications of insect symbionts in biotechnology. *Applied Microbiology and Biotechnology,* 100: 1567-1577.

Berasategui A, Axelson K, Nordlander G, Schmidt A, Borg-Karlson AK, Gershenzon J, Terenious O, Kaltenpoth M (2016) The gut microbiota of the pine weevil is similar across Europe and resembles that of other conifer-feeding beetles. *Molecular Ecology,* 25, 4014-4031.

Berenbaum M. (1980) Adaptive significance of midgut pH in larval Lepidoptera. *American Nat*uralist, 115: 138–146.

Berryman AA (1976) Theoretical explanation of mountain pine beetle dynamics in lodgepole pine forests. *Environmental Entomology*, 5, 1225–1233.

Bleiker KP, Six DL (2007) Dietary benefits of fungal associates to an eruptive herbivore: potential implications of multiple associates on host population dynamics. *Environmental Entomology*, 36, 1384–1396.

Boone CK, Keefover-Ring K, Mapes AC *et al.* (2013) Bacteria associated with a tree-killing insect reduce concentrations of plant defense compounds. *Journal of Chemical Ecology*, 39, 1003–1006.

Borg-Karlson AK, Nordlander G, Mudalige A, Nordenhem H, Unelius CR (2006) Antifeedants in the feces of the pine weevil *Hylobius abietis*: identification and biological activity. *Journal of Chemical Ecology*, 32, 943.

Bourtzis K (2008) *Wolbachia*-based technologies for insect pest population control. *Advances in Experimental Medicine and Biology,* 627:104–113.

Bourtzis K, Dobson SL, Xi Z, Rasgon JL, Calvitti M, Moreira LA, Bossin HC, Moretti R, Baton LA, Hughes GL, Mavingui P, Gilles JR (2014) Harnessing mosquito-*Wolbachia* symbiosis for vector and disease control. *Acta Tropica* 132(Suppl): S150–S163.

Bright M, Bulgheresi S (2010) A complex journey: transmission modes of microbial symbionts. *Nature Reviews Microbiology*, 8: 218-230.

Broderick NA, Raffa KF, Goodman RM, Handelsman J (2004) Census of the bacterial community of the gypsy moth larval midgut by using culturing and culture-independent methods. *Applied and Environmental Microbiology*, 70, 293–300.

Broderick NA, Buchon N, Lemaitre B (2014) Microbiota-induced changes in Dr*osophila melanogaster* host gene expression and gut morphology. *mBio* 5, 3 e01117-14.

Brune A, Dietrich C (2015). The gut microbiota of termites: digesting the diverstiy in the light of ecology and evolution. *Annual Review in Microbiology*, 69: 145-166.

Caspi-Fluger A, Inbar M, Mozes-Daube N, Katzir N, Portnoy V, Belausov E, Hunter MS, Zchori-Fein E (2012). Horizontal transmission oft he insect symbiont *Rickettsia* is plant-mediated. *Proceedings oft he Royal Society of London-B Biological Sciences,* 279: 1791-1796.

Ceja-Navarro JA, Vega FE, Karaoz U, Hao Z, Jenkins S, Lim HC, Kosina P, Infante F, Northen TR, Brodie EL. (2015) Gut microbiota mediate caffeine detoxification in the primary insect pest of coffee. *Nature. Communications,* **6**, 7618.

Cirimotich CM, Dong Y, Clayton AM, Sandiford SL, Souza-Neto JA, Mulenga M, Dimopoulos G(2011a) Natural microbe-mediated refractoriness to *Plasmodium* infection in *Anopheles gambiae*. *Science*, 332: 855–858.

Cirimotich C.M., Ramirez J.L., Dimopoulos G (2011b) Native microbiota shape insect vector competence for human pathogens. *Cell, Host and Microbe*,, 10: 307–310.

Chien A, Edgar DB, Trela JM (1976) Deoxyribonucleic acid polymerase from the extreme thermophile *Thermus aquaticus. Journal of Bacteriology,* 127: 1550-1557.

Chung SH, Rosa C, Scully ED, Peiffer M, Tooker JF, Hoover K, Luthe DS, Felton GW (2013) Hervivore exploits orally secreted bacteria to suppress plant defenses. *Proceedings of the National Academy of Sciences of the United States of America,* 110, 39: 15728-15733.

Clark, TM (1999) Evolution and adaptive significance of larval midgut alkalinization in the insect superorder *Mecopterida*. *Journal of Chemical Ecology,* 25: 1945.

Codella SG, Raffa KF (1995) Contributions of female oviposition patterns and larval behavior to group defense in conifer sawflies (Hymenoptera, Diprionidae). *Oecologia*, 103, 24–33.

Colman DR, Toolson EC, Takacs-Vesbach CD (2012) Do diet and taxonomy influence insect gut bacterial communities? *Molecular Ecology*, 21, 5124–5137.

Cook SP, Hain FP 1988 Toxicity of host monoterpenes to *Dendroctonus frontalis* and *Ips calligraphus* (Coleoptera: Scolytidae). *Journal of Entomological Sci*ences, 23 287–292.

Delalibera I, Vasanthakumar A, Burwitz BJ *et al.* (2007) Composition of the bacterial community in the gut of the pine engraver, *Ips pini* (Say) (Coleoptera) colonizing red pine. *Symbiosis*, 43, 97–104.

Despres L, David JP, Gallet C (2007). The evolutionary ecology of insect resistance to plant chemicals. *Trends in Ecology and Evolution,* 22, 6: 298-307.

Deneubourg JL, Gregoire JC, Lefort E (1990) Kinetics of larval gregarious behavior in the bark beetle *Dendroctonus micans* (Coleoptera, Scolytidae). *Journal of Insect Behavior*, 3, 169– 182.

DiGuistini S, Wang Y, Liao NY *et al.* (2011) Genome and transcriptome analyses of the mountain pine beetle-fungal symbiont *Grosmannia clavigera*, a lodgepole pine pathogen. *Proceedings of the National Academy of Sciences of the United States of America*, 108, 2504–2509.

Dillon RJm Vennard CT, Buckling A, Charnley AK (2005) Diversity of locust gut bacteria protects against pathogen invasion. *Ecology Letters,* 8, 1291-1298.

Dillon RJ & Dillon VM (2004) The gut bacteria of insects: nonpathogenic interactions. *Annual Review in Entomology,* 49:7.

Dohet L, Grègoire JC, Berasategui A, Kaltenpoth M, Biedermann PHW (2016). Bacterial and fungal symbionts of parasitic *Dendroctonus* bark beetles. *FEMS Microbiology and Ecol*ogy, doi: 10.1093/femsec/fiw129.

Douglas A.E. (2009) The microbial dimension in insect nutritional ecology. *Functional Ecology*, 23, 38-47.

Dowd, P.F., (1989) In situ production of hydrolytic detoxifying enzymes by symbiotic yeasts in the cigarette beetle (Coleoptera: Anobiidae). *Journal of Economic Entomology* 82, 396–400.

Dowd PF & Shen SK (1990) The contribution of symbiotic yeast to toxin resistance of the cigarette beetle (*Lasioderma serricorne*). *Entomologia Experimentalis et Applicata,* 56: 241–248.

Durvasula RV, Gumbs A, Panackal A, Kruglov O, Aksoy S, Merrifield RB, Richards FF, Beard CB (1997) Preventionof insect-borne disease: an approach using transgenic symbiotic bacteria. *Proceedings of the National Academy of Sciences of the United States of America*, 94: 3274–3278.

Durvasula RV, Gumbs A, Panackal A, Kruglov O, Taneja J, Kang AS, Cordon-Rosales C, Richards FF, Whitham RG, Beard CB (1999) Expression of a functional antibody fragment in the gut of *Rhodnius prolixus* via transgenic bacterial symbiont *Rhodococcus rhodnii*. *Medical and Veterinary Entomology,* 13: 115–119.

Eichler S, Schaub GA (2002) Development of symbionts in triatomine bugs and the effects of infections with trypanosomatids. *Experimental Parasitol*ogy, 100: 12-27.

Ekman R (1980) Geranylgeranyl esters in Norway spruce wood. *Phytochemistry* 19: 321–322

Engel P., Moran N. (2013) The gut microbiota of insects - diversity in structure and function. *FEMS Microbiology Rev*iews, 37: 699-735.

Farrell BD (1998) 'Inordinate fondness' explained: why are there so many beetles? *Science* 281:555–59.

Fäldt J, Sjodin K, Persson M, Valterova I, Borg-Karlson AK. (2001). Correlations between selected monoterpene hydrocarbons in the xylem of six *Pinus* (Pinaceae) species. *Chemoecology,* 11: 97–106.

Feltrer R, Alvarez-Rodriguez ML, Barreiro C, Godio RP, Coque JJR (2010) Characterization of a novel 2,4,6-trichlorophenol-inducible gene encoding chlorophenol O-methyltransferase from *Trichoderma longibrachiatum* responsible for the formation of chloroanisoles and detoxification of chlorophenols. *Fungal Genetics and Biology*, 47, 458–467.

Flórez LV, Biedermann PHW, Engl T, Kaltenpoth M (2015) Defensive symbioses of animals with prokaryotic and eukaryotic microorganisms. *Natural Product Reports* 32:904–936.

Forsgren E, Olofsson TC, Vasquez A & Fries I (2009) Novel lactic acid bacteria inhibiting *Paenibacillus* larvae in honeybee larvae. *Apidologie* 41:99–108.

Francois IEJA, De Bolle MFC, Dwyer G, Goderis IJWM, Woutors PFJ, Verhaert PD, Proost P, Schaaper WMM, Cammue BPA, Broekaert WF (2002) Transgenic expression in *Arabidopsis* of a polyprotein construct leading to production of two different antimicrobial proteins. *Plant Physiology,* 128:1346–1358.

Funari SS, Prades J, Escribá PV, Barceló F (2005) Farnesol and geranylgeraniol modulate the structural properties of phosphatidylethanolamine model membranes. *Molecular Membrane Biology,* 22: 303–311.

Gratshev VG, Zherikhin VV (2003) in *Proceedings of the 2nd Congress on Palaeoentomology*, eds Krzeminska E, Krzeminski W (Krakow, Poland), *Acta Zoologica Cracov* 46 *Suppl.* pp129–138.

Gregoire JC, Braekman JC, Tondeur A (1981) Chemical communication between the larvae of *Dendroctonus micans* Kug (Coleoptera: Scolytidae). *Les colloques de L'INRA, 7 Les mediateurs chimiques*, 253–257.

Grunwald S, Pilhofer M, Holl W. (2010) Microbial associations in gut systems of wood- and bark-inhabiting longhorned beetles (Coleoptera: Cerambycidae) *Systematic and Applied Microbiology,* 33:25–34.

Haeder S, Wirth R, Herz H, Spiteller D (2009) Candicidin-producing Streptomyces support leaf-cutting ants to protect their fungus garden against the pathogenic fungus *Escovopsis*. *Proceedings of the National Academy of Sciences of the United States of America*, 106:4742–4746.

Hammer TJ, Bowers MD (2015). Gut microbes may facilitate insect herbivory of chemically defended plants. *Oecologia*. 179: 1-14.

Helmus, MR, Dussourd DE (2005) Glues or poisons: which triggers vein cutting by monarch caterpillars? *Chemoecology* 15, 45–49.

Hilgenboecker K, Hammerstein P, Schlattmann P, Telschow A, Werren JH (2008) How many species are infected with *Wolbachia*? – a statistical analysis of current data. *FEMS Microbiology Letters*, 281, 215–220.

Holmes NA, Innocent TM, Heine D, Bassam MA, Worsley SF, Trottmann F, Patrick EH, Yu DW, Murrell JC, Schiøtt M, Wilkinson B, Boomsma JJ, Hutchings MI. (2006) Genome analysis of two *Pseudonocardia* phylotypes associated with *Acromyrmex* leafcutter ants reveals their biosynthetic potential. *Frontiers in Microbiology*, 7 2073.

Holzinger, F. and Wink, M. (1996) Mediation of cardiac glycoside insensitivity in the Monarch butterfly (*Danaus plexippus*): Role of an amino acid substitution in the ouabain binding site of Na+, K+- ATPase. *Journal of Chemical Ecology,* 22, 1921–1937.

Hongoh Y, Deevong P, Inoue T *et al.* (2005) Intra- and interspecific comparisons of bacterial diversity and community structure support coevolution of gut microbiota and termite host. *Applied and Environmental Microbiology*, 71, 6590-6599.

Hosokawa T, Kikuchi Y, Shimada M, Fukatsu T (2007) Obligate symbiont involved in pest status of host insect. *Proceedings of the Royal Society B: Biological Science*,274, 1979-1984.

Huber DPW, Bohlmann J (2006) The role of terpene synthases in the direct and indirect defense of conifers against insect herbivory and fungal pathogens. In "Multigenic and Induced systemic resistance in plants". Pp 296-313.

Hulcr J, Adams AS, Raffa K, Hofstetter RW, Klepzig KD, Currie R (2012) Presence and diversity of Streptomyces in *Dendroctonus* and sympatric bark beetle galleries across North America. *Microbial Ecology*, 61:759–68.

Inoue T, Murashima K, Azuma JI, Sugimoto A, Slaytor M. (1997) Cellulose and xylan utilization in the lower termite *Reticulitermes speratus. Journal of Insect Science,* 43: 235–242.

Ivanov I.I., Atarashi K., Manel N. *et al.* (2009) Induction of intestinal Th17 cells by segmented filamentous bacteria. *Cell*, 139: 485–498.

Jackson DL, Jaroski V, Dixon AFG (1996) Resource partitioning and tolerance of monoterpenes in four species of spruce aphid. *Physiological Entomology* 21: 242–246.

Jassbi, AR, Gase K, Hettenhausen C, Schmidt A, and Baldwin IT (2008) Silencing geranylgeranyl diphosphate synthase in *Nicotiana attenuata* dramatically impairs resistance to *Manduca sexta. Plant Physiol*ogy, 146 974–986.

Kaltenpoth M, Gottler W, Herzner G, Strohm E (2005) Symbiotic bacteria protect wasp larvae from fungal infestation. *Current Biology,* 15:475– 479.

Kaltenpoth M, Roeser-Mueller K, Koehler S, Peterson A, Nechitaylo T, Stubblefield JW, Herzner G, Seger J, Strohm E (2014) Partner choice and fidelity stabilize co-evolution in a Cretaceous-age defensive symbiosis. *Proceedings of the National Academy of Sciences of the United States of America,* 111:6359–6364.

Kambris Z, Cook PE, Phuc HK, Sinkins SP (2009) Immune activation by life-shortening *Wolbachia* and reduced filarial competence in mosquitoes. *Science* 326:134–136.

Kapin MA, Ahmad S (1980) Esterases in larval tissues of gypsy moth, *Lymantria dispar* (L.): optimum assay conditions, quantification and characterization. *Insect Biochemistry,* 10: 331–337.

Keeling CI, Bohlmann J (2006) Diterpene resin acids in conifers. *Phytochemistry*, 67, 2415–2423.

Kikuchi Y, Hosokawa T, Fukatsu T (2007) Insect-microbe mutualism without vertical transmission: a stinkbug acquires a beneficial gut symbiont from the environment every generation. *Applied and Environmental Microbiology.,* 73: 4308–4316.

Kikuchi Y., Hayatsu M., Hosokawa T., Nagayama A., Tago K., Fukatsu T. (2012) Symbiont- mediated insecticide resistance. *Proceedings of the National Academy of Sciences of the United States of America,* 109: 8618–8622.

Kirkendall LR, Biedermann PHW, Jordal BH (2015) Evolution and diversity of bark and ambrosia beetles. In "Bark beetles: biology and ecology of native and invasive species". Academic press

Kleppe, K., Ohtsuka, E., Kleppe, R; Molineux, I., and Khorana, H.E. (1971) Studies on polynucleutides. XCVI Repair replication of short synthetic DNA's as catalyzed DNA polymerase. *Journal of Molecular Biology*. 56: 341-361.

Klepzig KD, Kruger EL, Smalley EB, Raffa KF (1995) Effects of biotic and abiotic stress on induced accumulation of terpenes and phenolics in red pines inoculated with bark beetle-vectored fungus. *Journal of Chemical Ecology,* 21: 601-626.

Kohl KD, Dearing MD (2012) Experience matters: prior exposure to plant toxins enhances diversity of gut microbes in herbivores. *Ecology Letters,* 15:1008–1015.

Kroiss J, Kaltenpoth M, Schneider B, Schwinger M-G, Hertweck C, Maddula RK, Strohm E, Svatoš A (2010) Symbiotic streptomycetes provide antibiotic combination prophylaxis for wasp offspring. *Nature Chemical Biology,* 6:261–263.

Lachowska D, Kajtoch L, Knutelski S (2010) Occurrence of *Wolbachia* in central European weevils: correlations with host systematics, ecology, and biology. *Entomologia Experimentalis et Applicata*, 135, 105–118.

Lagenheim JH (2003). Plant resins: chemistry, evolution, ecology and ethnobotany. Portland, Cambridge: Timber Press.

Langor DW, Williams DJM (1998) Life cycle and mortality of *Pissodes terminalis* (Coleoptera: Curculionidae) in lodgepole pine. *Canadian Entomologist*, 130:387–397.

Lawyer FC, Stoffel S, Saiki RK, Chang SY, Landre PA, Ahramson RD, Gelfand DH (1993) High-level expression, purification, and enzymatic characterization of full-length *Thermus aquaticus* DNA polymerase and a truncated form deficient in 5' to 3' exonuclease activity. *PCR Methods and Applications* 2: 275-287.

Lemaire M, Nagnan P, Clement JL, Lange C, Peru L, Basselier JJ (1990) Geranyllinalool (diterpene alcohol): an insecticidal component of pine wood and termites (Isoptera: Rhinotermitidae) in four European ecosystems. *Journal of Chemical Ecology,* 16: 2067–2079.

Ley RE, Peterson DA, Gordon JI (2006) Ecological and evolutionary forces shaping microbial diversity in the human intestine. *Cell*, 124, 837–848.

Ley RE, Hamady M, Lozupone C *et al.* (2008) Evolution of mammals and their gut microbes. *Science*, 320, 1647–1651.

Lidgard S, Crane PR (1990) Angiosperm diversification and Cretaceous floristic trends: A comparison of palynofloras and leaf macrofloras. *Paleobiology* 16:77–93.

Lindgren BS, Nordlander G, Birgersson G (1996) Feeding deterrence of verbenone to the pine weevil, *Hylobius abietis* (L.) (Col., Curculionidae). *Journal of Applied Entomology* 120: 397–403.

Litvak ME, Monson RK (1998) Patterns of induced and constitutive monoterpene production in conifer needles in relation to insect herbivory. *Oecologia* 114: 531-540.

Mason CJ, Hanshew AS, Raffa KF. (2016) Contributions by host trees and insect activity to bacterial communities in *Dendroctonus valens* (Coleoptera: Curculionidae) galleries, and their high overlap with other microbial assemblages of bark beetles. *Environmental Entomology,* 45:348–56.

Manninen AM, Tarhanen S, Vuorinen M, Kainulainen P (2002) Comparing the variation of needle and wood terpenoids in Scots pine provenances. *Journal of Chemical Ecology* 28: 211–228.

Marivaldi AE, Sequeira AS, O´Brien CW, Farrel BD (2002) Molecular and morphological phylogenetics of weevils (Coleoptera, Curculionoidea): Do niche shifts accompany diversification? *Systematic Biology*, 51: 761-785.

Martin DM, Gershenzon J, Bohlmann J (2003) Induction of volatile terpene biosynthesis and diurnal emission by methyl jasmonate in foliage of Norway spruce. *Plant Physiology,* 132: 1586–1599.

Martin VJJ, Mohn WW (2000) Genetic investigation of the catabolic pathway for degradation of abietane diterpenoids by *Pseudomonas abietaniphila* BKME-9*. Journal of Bacteriology*, 182 (13):3784-3793.

Martinson VG, Danforth BN, Minckley RL *et al.* (2011) A simple and distinctive microbiota associated with honey bees and bumble bees. *Molecular Ecology*, 20, 619–628.

Martinson VG, Moy J & Moran NA (2012) Establishment of characteristic gut bacteria during development of the honey bee worker. *Applied and Environmental Microbiology,* 78: 2830–2840.

Marty, M.A. and Krieger, R.I. (1984) Metabolism of uscharidin, a milkweed cardenolide, by tissue-homogenates of monarch butterfly larvae, *Danaus plexippus* L. *Journal of Chemical Ecology,* 10, 945–956.

McCullough DC, Wagner MR (1993) Defusing Host Defenses: *Ovipositional Adaptations of Sawflies to Plant Resins*. Academic Press, San Diego.

McMeniman CJ, Lane RV, Cass BN, Fong AWC, Sidhu M, Wang YF & O'Neill SL (2009) Stable introduction of a life-shortening *Wolbachia* infection into the mosquito *Aedes aegypti. Science* 323: 141–144.

Mita E, Tsitsimpikou C, Tsiveleka L, Petrakis PV, Ortiz A, Vagias C, Roussis V (2002) Seasonal variation of oleoresin terpenoids from *Pinus halepensis* and *Pinus pinea* and host selection of the scale insect *Marchalina hellenica* (Homoptera, Coccoidea, Margarodidae, Coelostonidiinae). *Holzforschung* 56: 572–578.

Morales-Jimenez J, Zuniga G, Villa-Tanaca L, Hernandez-Rodriguez C (2009) Bacterial community and nitrogen fixation in the red turpentine beetle, *Dendroctonus valens* LeConte (Coleoptera: Curculionidae: Scolytinae). *Microbial Ecology*, 58, 879–891.

Morales-Jiménez J, Zúñiga G, Ramírez-Saad HC, Hernández-Rodríguez C (2012) Gut-associated bacteria throughout the life cycle of the bark beetle *Dendroctonus rhizophagus* Thomas and Bright (Curculionidae: Scolytinae) and their cellulolytic activities. *Microbial Ecology,* 64(1):268-78.

Morales-Jimenez J, Vera-Ponce de León A, García-Domínguez A, Martínez-Romero E, Zúñiga G, Hernández-Rodríguez C (2013). Nitrogen-fixing and urolytic bacteria associated with the gut of *Dendroctonus rhizophagous* and *Dendroctonus valens* (Curculionidae: Scolytinae). *Microbial Ecology* 66, 200-210.

Muegge BD, Kuczynski J, Knights D etal. (2011) Diet drives convergence in gut microbiome functions across mammalian phylogeny and within humans. *Science*, 332, 970–974.

Nagel R, Berasategui A, Paetz C, Gershenzon J, Smidth A (2014). Overexpression of an isoprenyl diphosphate synthase in spruce leads to unexpected terpene diversion products that function in plant defense. *Plant Phys*iology, 164: 555-569.

Nikoh N, Hosokawa T, Oshima K, Hattori M, Fukatsu T (2011) Reductive evolution of bacterial genome in insect gut environment. *Genome Biology and Evolution*, 3, 702–714.

Nishida, R. (2002) Sequestration of defensive substances from plants by Lepidoptera. *Annual Review in Entomology,* 47, 57–92.

Nordlander G (1990) Limonene inhibits attraction to alpha-pinene in the pine weevils *Hylobius abietis* and *H. pinastri*. *Journal of Chemical Ecology* 16: 1307–1320.

Oh DC, Poulsen M, Currie CR, Clardy J (2009a) Dentigerumycin: a bacterial mediator of an ant-fungus symbiosis. *Nature Chemical Biology,* 5: 391–393.

Olofsson TC, Vásquez A (2008) Detection and identification of a novel lactic acid bacterial flora within the honey stomach of the honeybee *Apis mellifera*. *Current Microbiology,* 57:356–363.

Parr JC, Thurston R (1972) Toxicity of nicotine in synthetic diets to larvae of the tobacco hornworm. *Annals* of the *Entomological Society of America,* 65:1185–1188.

Phillips MA, Croteau RB (1999) Resin-based defenses in conifers. *Trends in Plant Science,* 4:184–190.

Pernice M, Simpson SJ, Ponton F (2014) Towards an integrated understanding of gut microbiota using insects as model systems. *Journal of Insect Physiology,*69: 12-18.

Raffa KF (2013) Terpenes tell different tales at different scales: glimpses into the chemical ecology of conifer – bark beetle – microbial interactions. *Journal of Chemical Ecology*, 40, 1–20.

Raffa KF, Berryman AA (1982) Physiological differences between lodgepole pines resistant and susceptible to the mountain pine beetle and associated microorganisms. *Environmental Entomology,* 11, 486–492.

Raffa KF, Smalley EB (1995) Interaction of pre-attack and induced monoterpene concentrations in host conifer defense against bark beetle-fungal complexes. *Oecologia* 102 285–295.

Ramadhar TR, Beemelmanns C, Currie CR, Clardy J (2014) Bacterial symbionts in agricultural systems provide a strategic source for antibiotic discovery. *Journal of Antibiotics,* 67:53–58.

Ren C, Webster P, Finkel SE & Tower J (2007) Increased internal and external bacterial load during *Drosophila* aging without life-span trade-off. *Cell Metabolism,* 6: 144–152.

Richards LA, Lampert EC, Bowers MD et al (2012) Synergistic effects of iridoid glycosides on the survival, development and immune response of a specialist caterpillar, *Junonia coenia* (Nymphalidae). *Journal of Chemical Ecology,* 38:1276–1284.

Robert CAM, Frank DL, Leach KA, Turlings TCJ, Hibbard BE, Erb M (2013) Direct and indirect plant defenses are not suppressd by endosimbyionts of a specialist root herbivore. *Journal of Chemical Ecology,* 39:507-515.

Ryu JH, Ha EM & Lee WJ (2010) Innate immunity and gut-microbe mutualism in *Drosophila*. *Developmental and Comparative Immunology,* 34:369–376.

Saiki RK, Gelfand DH, Stoffel S, Scharf SJ, Higuchi R, Horn GT, Mullis KB, Erlich HA (1988) Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* 239: 487-491.

Salem H., Kreutzer E., Sudakaran S., Kaltenpoth M. (2013) Actinobacteria as essential symbionts in firebugs and cotton stainers (Hemiptera, Pyrrhocoridae). *Environmental Microbiology*, 15, 1956-1968.

Salem H, Bauer E, Strauss AS, Vogel H, Marz M, Kaltenpoth M (2014) Vitamin supplementation by gut symbionts ensures metabolic homeostasis in an insect host. *Proceedings of the Royal Society B: Biological Sciences* 281: 20141838.

Salem H, Florez L, Gerardo N, Kaltenpoth M (2015) An out-of-body experience: the extracellular dimension for the transmission of mutualistic bacteria in insects. *Proceedings of the Royal Society B: Biological Sciences* 282: 20142957.

Sanders JG, Powel S, Kronauer DJ, Vasconcelos HL, Frederickson ME, Pierce NE (2014) Stability and phylogenetic correlation in gut microbiota: lessons from apes and ants. *Molecular Ecology*. 6: 1268-1283.

Schloss PD, Delalibera I Jr, Handelsman J & Raffa KF (2006) Bacteria associated with the guts of two wood-boring beetles: *Anoplophora glabripennis* and *Saperda vestita* (Cerambycidae). *Environmental Entomology* 35: 625–629.

Sender R, Fuchs S, Milo R (2016) Revised Estimates for the Number of Human and Bacteria Cells in the Body. *PLoS Biology,* 14(8): e1002533.

Sharon G, Segal D, Ringo JM, Hefetz A, Zilber-Rosenberg I, Rosenberg E. (2010) Commensal bacteria play a role in mating preference of *Drosophila melanogaster*. *Proceedings of the National Academy of Sciences of the United States of America,* 107, 20051-20056.

Six DL, Paine TD (1998) Effects of mycangial fungi and host tree species on progeny survival and emergence of *Dendroctonus ponderosae* (Coleoptera: Scolytidae). *Environmental Entomology*, 27, 1393–1401.

Six DL, Wingfield MJ (2011) The role of phytopathogenicity in bark beetle-fungus symbioses: a challenge to the classic paradigm. *Annual Review in Entomology* 56, 255-272.

Smith DJ, Martin VJ, Mohn WW (2004) A cytochrome P450 involved in the metabolism of abietane diterpenoids by *Pseudomonas abietaniphila* BKME-9. *Journal of Bacteriology,* 186:3631-3639.

Smith DJ, Park J, Tiedje J, Mohn WW (2007) A large gene cluster in *Burkholderia xenovorans* encoding abietane diterpenoid catabolism. *Journal of Bacteriology,* 189:17, 6195-6204.

Stansly, P. A. and Cate, J. R. 1984. Discrimination by ovipositing boll weevils (Coleoptera: Curculionidae) against previously infested Hampea (Malvaceae) flower buds. *Environmental Entomology,* 13:1361–1365.

Stecher B, Hardt WD (2011) Mechanisms controlling pathogen colonization of the gut. *Current Opinion in Microbiology*., 14: 82–91.

Sudakaran S, Salem H, Kost C, Kaltenpoth M (2012) Geographical and ecological stability of the symbiotic mid-gut microbiota in European firebugs, *Pyrrhocoris apterus* (Hemiptera, Pyrrhocoridae). *Molecular Ecology*, 21: 6134–6151.

Tomlin ES, Antonejevic E, Alfaro RI, Borden JH (2000) Changes in volatile terpene and diterpene resin acid composition of resistant and susceptible white spruce leaders exposed to simulated white pine weevil damage. *Tree Physiology* 20: 1087–1095.

Toju H, Fukatsu T (2011) Diversity and infection prevalence of endosymbionts in natural populations of the chestnut weevil: relevance of local climate and host plants. *Molecular Ecology*. 20: 853-868.

Trapp S, Croteau R (2001) Defensive resin biosynthesis in conifers. *Annual Review in Plant Physiology and Plant Molecular Biology,* 52:689–724.

Vasanthakumar A, Delalibera I, Handelsman J *et al.* (2006) Characterization of gut-associated bacteria in larvae and adults of the southern pine beetle, *Dendroctonus frontalis* Zimmermann. *Environmental Entomology*, 35, 1710–1717.

Vicente CSL, Nascimento FX, Espada M *et al.* (2013) Characterization of bacterial communities associated with the pine sawyer beetle *Monochamus galloprovincialis*, the insect vector of the pinewood nematode *Bursaphelenchus xylophilus*. *FEMS Microbiology Letters*, 347, 130–139.

Vilanova C, Marín M, Baixeras J, Latorre A, Porcar M (2014) Selecting microbial strains from pine tree resin: biotechnological applications from a terpene world. *PLoS ONE* 9(6): e100740.

Vollaard EJ, Clasener HA (1994) Colonization resistance. *Antimicrobial Agents and Chemotherapy*, 38: 409–414.

Wainhouse D, Ashburner R, Boswell R (2001) Reproductive development and maternal effects in the pine weevil *Hylobius abietis*. *Ecological Entomology,* 26 655-61.

Wallace DR, Sullivan CR (1985) The white pine weevil, *Pissodes strobi* (Coleoptera: Curculionidae): a review emphasizing behavior and development in relation to physical factors. *Proceedings of the Entomological Society of Ontario,* 116:39–62.

Wang Y, Lim L, DiGuistini S *et al.* (2013) A specialized ABC efflux transporter GcABC-G1 confers monoterpene resistance to *Grosmannia clavigera*, a bark beetle-associated fungal pathogen of pine trees. *New Phytologist*, 197, 886–898.

Werner RA. (1995) Toxicity and repellency of 4-allylanisole and monoterpenes from white spruce and tamarack to the spruce beetle and eastern larch beetle (Coleoptera: Scolytidae). *Environental. Entomology* 24 372–379.

Werren JH (1997) Biology of *Wolbachia*. *Annual Review in Entomology,* 42:587– 609.

Werren JH, Baldo L, Clark ME (2008) Wolbachia: master manipulators of invertebrate biology. *Nature Reviews Microbiology*, 6, 741–751.

Wen X, Kuang, Y, Shi M, Li H, Luo Y, Deng R (2004) Biology of *Hylobitelus xiaoi* (Coleoptera: Curculionidae), a new pest of slash pine, *Pinus elliottii*. *Journal of Economic Entomology*, 97:1958–1964.

Whittome B, Graham RI, Levin DB (2007) Preliminary examination of gut bacteria from *Neodiprion abietis* (Hymenoptera: Diprionidae) larvae. *Journal of the Entomological Society of Ontario*, 138, 49-63.

Whitsitt M (1933) Vitamin B(B1) and G(B2) content of cotton-seed products. *Industrial and Engineering Chemistry Research,* 25: 1169–1171.

Wöll S, Kim SH, Greten HJ, Efferth T (2013) Animal plant war- fare and secondary metabolite evolution. *Natural Products and Bioprospecting*, 3, 1–7.

Xu LT, Lu M, Sun JH (2015) Invasive bark beetle associated microbes degrade a host defensive monoterpene. *Insect Science*. 23 183-90.

Yong E (2016) Terms and conditions apply. *In: I contain multitudes*. HarperCollins Publishers Pp: 77-102.

Yun JH, Roh SW, Whon TW, Jung MJ, Kim MS, Park DS, Yoon C, Nam YD, Kim YJ, Choi JH, Kim JY, Shin NR, Kim SH, Lee WJ, Bae JW (2014) Insect gut bacterial diversity determined by environmental habitat, diet, developmental stage, and phylogeny of host. *Applied and Environmental Microbiology,* 80: 5254-5264.

Zhang H, Ye H, Haack RA, Langor, DW (2004) Biology of *Pissodes yunnanensis* (Coleoptera: Curculionidae), a pest of Yunnan pine in southwestern China. *Canadian Entomologist*,136:719–726.

Zouache K, Raharimalala FN, Raquin V *et al.* (2011) Bacterial diversity of field-caught mosquitoes, *Aedes albopictus* and *Aedes aegypti*, from different geographic regions of Madagascar. *FEMS Microbiology Ecology*, 75, 377–389.

SUMMARY

The gut microbiome of insects is known to play essential roles in host's fitness through a variety of functions including nutritional supplementation of diets, influencing development, and mediating parasite and pathogen resistance. In this thesis, we have explored the role that gut microbes may play in the degradation of plant secondary metabolites that are toxic to insects, focusing primarily on the pine weevil (*Hylobius abietis*) and terpenoids.

Characterization of the gut bacterial community of the pine weevil revealed a relatively stable microbiota at higher taxonomical levels (e.g. family and genus) across different locations in Europe, especially within the Enterobacteriaceae family. Despite showing some variation between locations, the pine weevil harbors a "core microbiota" composed of close relatives of *Rahnella* sp, *Erwinia* sp and *Serratia* sp.

Likewise, characterization of the gut microbiome of the parasitic or near-parasitic bark beetles *Dendroctonus valens, D. micans,* and *D. punctatus* reveal that they are also dominated by Enterobacteriacea. Additionally, all bacterial genera characterized as "core microbiota" in the pine weevil are also present in the microbiome of these bark beetles. Furthermore, our meta-analysis comparing the gut microbiomes of different conifer feeding beetle species and closely related beetles feeding on non-coniferous diets reveals that those sharing a food source harbor more similar bacterial communities than those that do not.

An *in silico* inference of the bacterial metagenome of the pine weevil revealed the putative presence of a gene cluster involved in diterpene degradation, particularly within members of the Enterobacteriaceae family. Altogether, these data suggest that the gut microbiota of the pine weevil and bark beetles might be acquired from the environment and may play an important ecological role for the insect, most likely related to nutrition.

In order to assess whether the role that the gut microbiome of the pine weevil plays for its host we performed both *in vivo* and *in vitro* bioassays. Our results demonstrate that after digestion, the concentration of diterpenes in feces of the pine weevil is reduced by 80% relative to ingested material. We also observed a significant reduction in the concentration of the diterpene dehydroabietic acid in liquid media in the presence of the pine weevil gut bacterial community, but not in their absence, suggesting that degradation of diterpenes may be mediated by symbionts. Our bioassays confirm the ability of the gut microbiome of the pine weevil to degrade diterpenes, given that antibiotic treatment disrupts the bacterial community of *H. abietis* and impairs its ability to digest terpenoids relative to individuals reared on their natural diet. Furthermore, reinfection of the insect with their native microbial assembly rescues the ability to degrade terpenoids.

This microbe-mediated degradation of terpenoids in the insect gut is consistent with our ability to annotate an almost complete gene cluster involved in diterpene degradation per our metagenomic analysis of the pine weevils' bacterial community.

Furthermore, bioassays showed that insects reared on an artificial diet amended with diterpenes were more fecund than antibiotic-treated ones, suggesting that microbe-driven catabolism of terpenes can enhance host fitness.

In this thesis, we have aimed to contribute to current knowledge on symbiont-mediated degradation of plant secondary metabolites.

ZUSSAMENFASSUNG

Die Gesamtheit der Darmbakterien von Insekten ist bekannt dafür, durch eine Vielzahl von Funktionen wie beispielsweise der Nahrungsaufarbeitung, der Beeinflussung der Wirtsentwicklung als auch der Vermittlung von Pathogen- als auch Parasitenresistenzen, eine entscheidende ökologische Funktion zur Fitness des Wirtes beizutragen.

Innerhalb der vorliegenden Arbeit wurde die Rolle der Darmbakterien beim Abbau von pflanzlichen Sekundärmetaboliten, die eigentlich giftig für das Insekt sind näher untersucht. Dabei stand der Große Braune Rüsselkäfers (*Hylobius abietis*) im Zusammenspiel mit pflanzlichen Terpenen im Mittelpunkt unserer Untersuchungen.

Eine Charakterisierung aller verschiedenen Arten von Darmbakterien des Rüsselkäfers ergab speziell in höheren taxonomischen Ordnungen (z. B. Familie und Geschlecht) eine vergleichbare Zusammensetzung innerhalb der Familie der Enterobakterien an verschiedenen Orten Europas. Unabhängig von einigen kleineren Unterschieden wird die bakterielle Zusammensetzung der Darmbakterien des Rüsselkäfers jeweils von den Arten Rahnella, Erwinia als auch Serratia dominiert.

Die durch uns erhaltenen Ergebnisse konnten durch vergleichbare Experimente anderer Arbeitsgruppen betätigt werden. So setzen sich die Darmbakterien der parasitischen Borkenkäfer *Dendroctonus valens, D. micans* und *D. punctatus* ebenso hauptsächlich aus Enterobakterien zusammen und werden in gleichem Umfang von den oben genannten Bakterienstämmen vorherrschend besiedelt. Ebenso ergab eine Analyse der Darmbakterien von Spezies, die an Koniferen fressen bzw. nicht fressen, dass jeweils vergleichbare Bakterienstämme nur in den Arten vorkommen, die auch vergleichbare Nahrung bevorzugen.

Zusammenfassend wäre also festzustellen, dass die Zusammensetzung der Gesamtheit der Darmbakterien sich aus dem umliegenden Habitat oder der aufgenommenen Nahrung ergibt und demzufolge wahrscheinlich einer bedeutende ökologische Funktion für das Insekt ausübt.

Um zu überprüfen ob die Zusammensetzung der einzelnen Bakterienarten auch eine Rolle bei der Überwindung von pflanzlichen Abwehrreaktionen, zu denen die Akkumulation von Terpenen zu zählen ist, spielen könnte, wurden *in-vitro*-Versuche durchgeführt. Dabei wurde eine signifikante Verringerung des Diterpens Dehydroabietinsäure in Flüssigmedium nur in der Gegenwart und nicht bei Abwesenheit von Darmbakterien festgestellt. Daher ist anzunehmen, dass der Terpenabbau auch *in vivo* innerhalb des Insektendarms abläuft. In weiterführenden Experimenten wurde festgestellt, dass eine Behandlung des Käfers mit Antibiotika diesen Abbau aufhebt.

Weiterhin konnte der durch Bakterien gesteuerte Abbau von Terpenen durch die Identifizierung eines Genclusters, was nachweislich am Abbau von Terpenen in anderen Insekten beteiligt sei, innerhalb der im Insektendarm enthaltenden Bakteriengenome belegt werden.

Ebenso konnte in Bioassays gezeigt werden, dass Käfer, die ausschließlich an einer mit Diterpenen versetzten künstlichen Diät fraßen, in der Folge fruchtbarer waren als jene, die keine Diterpene mit der Nahrung aufnahmen, was eine erhöhte ökologische Fitness dieser Käfer impliziert.

Mit dieser Dissertation erhoffen wir wichtige Erkenntnisse zum tieferen Verständnis des durch Bakteriensymbionten vermittelten Abbaus pflanzlicher Sekundärmetabolite beizutragen.

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Berasategui A., Lundhagen A., Nilsson L., Semberg E., Powell J., Terenius O. Bacteria associated with larval habitats of *Aedes* mosquitoes. *In prep*.

Berasategui A., Salem, H., Paetz, C., Schmidt, A., Kaltenpoth, M., Gershenzon J. Conserved microbiota in conifer-feeding beetles mediates the degradation of host plant defenses in the pine weevil. *In prep*.

Li H., Li T., Berasategui A., Zhang X., Li C., Xiao Z., Li X. Gut region and host species shape the diversity, network interactions and ecosystem stability of bacterial communities in the pika gastrointestinal tract. *Submitted*.

Dohet L., Gregoire J.C., Berasategui A., Kaltenpoth M., Biedermann P.H.W. (2016) Bacterial and fungal symbionts of parasitic *Dendroctonus* bark beetles. *FEMS Microb. Ecol.* DOI: 10.1093/femsec/fiw129

Berasategui, A., Axelsson, A., Norlander, G., Borg-Karlson, A-K., Schmidt, A., Gershenzon, J., Terenius, O. and Kaltenpoth, M. (2016) The gut microbiota of the pine weevil is similar across Europe and resembles that of other conifer-feeding beetles. *Molecular Ecology* 25, 4014–4031.

Berasategui, A., Shukla, S., Salem, H. and Kaltenpoth, M. (2015) Potential applications of insect symbionts in biotechnology. *Applied Microbiol. Biotech.* 100: 1567-1577.

Nagel, R., Berasategui, A., Paetz, C., Gershenzon, J., Schmidt, A. (2014). Overexpression of an isoprenyl diphosphate synthase in spruce leads to unexpected terpene diversion products that function in plant defense. *Plant Physiology*, 164(2), 555-569.

ORAL PRESENTATIONS

Berasategui A. (2016) Conserved microbiota in a herbivorous beetle mediates the degradation of host plant defenses. Talk presented at Evolution 2016, Austin, TX., USA.

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EIGENSTÄNDIGKEITSERKLÄRUNG

Entsprechend der geltenden, mir bekannten Promotionsordnung der Biologisch-Pharmazeutischen Fakultät der Friedrich-Schiller-Universität Jena erkläre ich, daß ich die vorliegende Dissertation eigenständig angefertigt und alle von mir benutzten Hilfsmittel und Quellen angegeben habe. Personen, die mich bei der Auswahl und Auswertung des Materials sowie bei der Fertigstellung der Manuskripte unterstützt haben, sind am Beginn eines jeden Kapitels genannt. Es wurde weder die Hilfe eines Promotionsberaters in Anspruch genommen, noch haben Dritte für Arbeiten, welche im Zusammenhang mit dem Inhalt der vorliegenden Dissertation stehen, geldwerte Leistungen erhalten. Die vorgelegte Dissertation wurde außerdem weder als Prüfungsarbeit für eine staatliche oder andere wissenschaftliche Prüfung noch als Dissertation an einer anderen Hochschule eingereicht.

Aileen Berasategui

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